UNIVERSIDAD AUTÓNOMA DE MADRID

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Departamento de Química Física Aplicada

LIBERACIÓN DE PÉPTIDOS MULTIFUNCIONALES DURANTE LA DIGESTIÓN GASTROINTESTINAL SIMULADA DE PROTEÍNAS DE QUINUA (*Chenopodium quinoa* Willd.) Y AMARANTO (*Amaranthus caudatus*)

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INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN (CSIC-UAM)

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A Dios, mi esposa e hijos

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“La ciencia se compone de errores, que a su vez son los pasos hacia la verdad”

Julio Verne

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RESUMEN

En la presente Tesis Doctoral se ha llevado a cabo el estudio de la quinua (*Chenopodium quinoa* Willd.) y amaranto (*Amaranthus caudatus*) como fuentes de péptidos biactivos. Por un lado, se han estudiado las modificaciones que sufren las proteínas alimentarias durante la digestión gastrointestinal y, por otro, se han evaluado diferentes actividades biológicas, identificando los péptidos potencialmente responsables de dichas actividades.

Inicialmente, se llevó a cabo la optimización del proceso de extracción de los concentrados proteicos de quinua y amaranto evaluando el efecto, tanto del pH de solubilización como el de precipitación de las proteínas, sobre el rendimiento del proceso y la concentración de proteína obtenida. En el caso del concentrado de proteína de quinua, el mayor rendimiento (6,3%) se obtuvo precipitando a pH 4,0 las proteínas presentes en la harina desengrasada obtenida a partir de este pseudocereal. Tras el análisis mediante electroforesis en gel de poliacrilamida nativa (PAGE-nativa) y con agentes desnaturalizantes (PAGE-SDS) pudieron visualizarse las proteínas mayoritarias contenidas en el concentrado de quinua. La banda con peso molecular de 60 kDa correspondió a la globulina 7S, mientras que las bandas de peso molecular 33-36 kDa y 20-22 kDa correspondieron a las dos subunidades de la globulina 11S. La banda de peso molecular inferior a 15,4 kDa correspondió a las albúminas. En el caso del amaranto, se llevó a cabo la extracción solubilizando las proteínas en condiciones alcalinas a dos pHs (8,0 y 12,0) y precipitando dichas proteínas a diferentes pHs, empleando agua o NaCl como solventes. El mayor rendimiento se obtuvo con la solución salina, aunque la necesidad de llevar a cabo un proceso de diálisis posterior limita el uso de dicha solución como solvente. La caracterización del perfil proteico mediante PAGE-SDS reveló similitud con el perfil de las proteínas de quinua, con una banda de peso molecular 50 kDa, correspondiente a la globulina 7S y varias bandas comprendidas entre 36-38 kDa y 18-20 kDa, que correspondieron a las dos subunidades, ácida y básica, de la globulina 11S. Las albúminas se visualizaron en aquellas bandas con pesos moleculares inferiores a 14,4 kDa.

Resumen

Dado que en los últimos años, las tasas de incidencia y mortalidad de desórdenes crónicos, como el síndrome metabólico, los trastornos cardiovasculares y neurológicos y el cáncer han aumentado de forma notable en nuestra sociedad, se planteó evaluar el papel de las proteínas de quinua y amaranto, tras su digestión gastrointestinal simulada, como fuente de péptidos multifuncionales, centrándose en el estudio de su actividad anti-diabética, antioxidante, anti-hipertensiva y citotóxica frente a células de cáncer de colon. Los concentrados proteicos de ambos pseudocereales fueron sometidos a un proceso de digestión gastrointestinal simulando las condiciones fisiológicas, con incubación secuencial con pepsina y pancreatina. Para ambas especies, la digestión con pepsina hidrolizó de forma parcial las proteínas, que fueron degradadas completamente tras la acción de la pancreatina. Con el objetivo de separar las fracciones peptídicas mayor y menor a 5 kDa, se llevó a cabo la ultrafiltración de los digeridos gástricos y gastroduodenales, evaluando las diferentes actividades biológicas tanto en los digeridos como en sus correspondientes fracciones. La actividad anti-diabética *in vitro* se evaluó midiendo la capacidad de los péptidos para inhibir la enzima dipeptidil peptidasa IV (DPP-IV), implicada en la degradación de las hormonas incretinas y las enzimas α-amilasa y α-glucosidasa, implicadas en la hidrólisis de carbohidratos. La actividad anti-hipertensiva se evaluó mediante el método *in vitro* de inhibición de la enzima convertidora de angiotensina (ECA), involucrada en la regulación de la presión arterial. La actividad antioxidante se determinó mediante dos métodos *in vitro*, evaluando la capacidad para neutralizar radicales peroxilo (ensayo ORAC) y para inhibir la peroxidación lipídica (ensayo TBARS). Por último, la actividad citotóxica se basó en la capacidad de los péptidos para inhibir la viabilidad de células de cáncer de colon, empleando tres líneas celulares diferentes (Caco-2, HT-29 y HCT-116).

En el caso de las proteínas de quinua, tanto los digeridos obtenidos durante la fase gástrica como los gastroduodenales presentaron todas las actividades biológicas determinadas, aunque los mayores efectos anti-diabéticos, antioxidantes y citotóxicos se determinaron en el digerido obtenido durante la fase duodenal. Los péptidos de pequeño tamaño fueron responsables de la actividad inhibidora de las enzimas DPP-IV,

α-amilasa y α-glucosidasa y de la actividad antioxidante, mientras que los péptidos contenidos en la fracción mayor a 5 kDa fueron los principales responsables de inhibir la viabilidad de las células de cáncer de colon. El digerido gastroduodenal se sometió a un fraccionamiento mediante HPLC a escala semi-preparativa, separándose tres fracciones que mostraron propiedades multifuncionales. El análisis mediante HPLC acoplado a espectrometría de masas en tándem permitió identificar 17 nuevos péptidos liberados a partir de las proteínas de quinua, potencialmente responsables de los efectos beneficiosos para la salud. Destacan las secuencias IQAEGGLT, DKDYPK y GEHGSDGNV, derivadas de la globulina 11S, cuyos efectos anti-diabéticos fueron confirmados.

Al igual que las proteínas de quinua, las de amaranto presentaron potencialidad como fuente de péptidos bioactivos tras su digestión gastrointestinal simulada. El digerido gástrico mostró una mayor capacidad de inhibición de la ECA, mientras que los mayores efectos antioxidantes, inhibidores de las enzimas DPP-IV y α-amilasa y de la viabilidad de las células Caco-2 se observaron en el digerido obtenido durante la fase duodenal. En dicho digerido gastroduodenal, el fraccionamiento y posterior análisis mediante HPLC-MS/MS permitió la identificación de 13 nuevos péptidos procedentes de las proteínas secuenciadas de esta especie vegetal, globulina 11S, albúmina 1 y poliamina oxidasa. Entre estos péptidos y tomando como base los estudios existentes en la literatura sobre la relación entre la estructura y las diferentes actividades biológicas pudo concluirse que las secuencias FLISCLL, SVFDEELS y DFIILE podrían ser consideradas como péptidos multifuncionales, ejerciendo actividad antioxidante y capacidad para inhibir la ECA y la α-amilasa. La presencia de estos péptidos y las posibles interacciones entre ellos podría explicar la actividad multifuncional ejercida por los digeridos de las proteínas de amaranto.

Recientemente, el modelo de pez cebra (*Danio rerio*) ha sido empleado de forma exitosa para estudiar la actividad antioxidante de péptidos derivados de proteínas alimentarias. Por lo tanto, se planteó su empleo para evaluar la actividad inhibidora de la peroxidación lipídica de péptidos procedentes de las proteínas de quinua y amaranto tras su digestión gastrointestinal *in vitro*. Los péptidos liberados durante la fase gástrica

presentaron efectos inhibidores de la peroxidación que alcanzaron el 75,2% en el digerido procedente de las proteínas de quinua. La actividad antioxidante aumentó de forma notable tras la hidrólisis con pancreatina hasta valores de inhibición del 82,1% y 77,6% para los digeridos gastroduodenales de proteína de quinua y amaranto, respectivamente. Estos valores fueron comparables al obtenido para el antioxidante sintético butilhidroxitoluol (BHT), usado como control positivo (87,1%).

Considerando todos los resultados en conjunto podemos confirmar el importante papel de las proteínas de quinua y amaranto como fuente de péptidos multifuncionales tras su digestión gastrointestinal, de modo que dichas proteínas podrían ser utilizadas como nuevos ingredientes de alimentos funcionales o nutracéuticos, con el fin de reducir las enfermedades asociadas a la diabetes, hipertensión arterial y el estrés oxidativo, incluido el cáncer. Además, los avances obtenidos en esta Tesis Doctoral permiten incrementar el valor nutricional y biológico ya reconocido para estos dos pseudocereales, cuya producción y consumo a nivel mundial se ha incrementado de forma notable en los últimos años.

LISTA DE ABREVIATURAS

BBI: inhibidor de proteasas Bowman-Birk

BHA: butilhidroxianisol

BHT: butilhidroxitoluol

DMT2: diabetes mellitus tipo 2

DPP-IV: dipeptidil peptidasa IV

ECA: enzima convertidora de angiotensina

FOSHU: alimentos para uso específico de salud

GIP: péptido insulinotrópico dependiente de la glucosa

GLP-1: péptido similar al glucagón tipo 1

GSH: glutatión reducido

HDL: lipoproteínas de alta densidad

HPLC-MS/MS: cromatografía de líquidos de alta eficacia acoplada a espectrometría de masas en tándem

IGF-1: factor de crecimiento insulínico tipo 1

KTI: inhibidor de tripsina Kunitz

LDL: lipoproteínas de baja densidad

LPS: lipopolisacárido

PAGE-SDS: electroforesis en gel de poliacrilamida con dodecilsulfato sódico

RCS: especies reactivas de cloro

RNS: especies reactivas de nitrógeno

ROS: especies reactivas de oxígeno

TBARS: sustancias reactivas al ácido tiobarbitúrico

t-BOOH: *tert*-butil hidroperóxido

OBJETIVOS Y PLAN DE TRABAJO

En los últimos años, la incidencia y mortalidad por distintos desórdenes crónicos ha aumentado de forma considerable en nuestra sociedad. El alto coste de las terapias existentes junto con los efectos adversos asociados a dichas terapias ha promovido el desarrollo de nuevas estrategias de prevención y/o control de los trastornos crónicos que sean eficaces y seguros para el consumidor. Estas estrategias se basan en los compuestos bioactivos presentes en fuentes naturales, destacando los péptidos liberados a partir de las proteínas alimentarias. Estos péptidos han centrado el interés de numerosos estudios por sus propiedades moduladoras de los diferentes sistemas del organismo, como el metabólico, cardiovascular, neurológico y de defensa. Aunque inicialmente fueron las proteínas animales las principales fuentes de péptidos bioactivos identificados, en los últimos años, la investigación se ha enfocado en las proteínas de origen vegetal debido a su sostenibilidad respecto a las proteínas animales y a la mayor preocupación de los consumidores por dietas saludables y equilibradas. Se buscan fuentes vegetales alternativas de péptidos bioactivos que permitan, por un lado, desarrollar nuevos alimentos funcionales y por otro, incrementar el valor nutricional y funcional de dichas especies vegetales.

Para establecer la relación existente entre un alimento y su efecto beneficioso para la salud es necesario conocer los procesos que tienen lugar desde la ingesta de dicho alimento hasta el efecto biológico ejercido por los compuestos contenidos en el mismo. En el caso de las proteínas, se considera esencial el seguimiento de los cambios que tienen lugar durante su digestión gastrointestinal, mediante el análisis, tanto de la degradación proteica producida por la acción de las enzimas digestivas como de la liberación de secuencias peptídicas potencialmente activas. Aunque los ensayos clínicos son la referencia en este tipo de análisis, sus implicaciones prácticas y éticas han limitado el uso de los estudios en humanos, por lo que es necesario disponer de modelos de digestión *in vitro* que simulen las condiciones fisiológicas.

Objetivo general

El objetivo principal de esta Tesis Doctoral es evaluar la potencialidad de las proteínas de quinua y amaranto como fuente de péptidos multifuncionales tras su

digestión gastrointestinal simulada. Para cumplir este objetivo se plantearon los siguientes objetivos específicos:

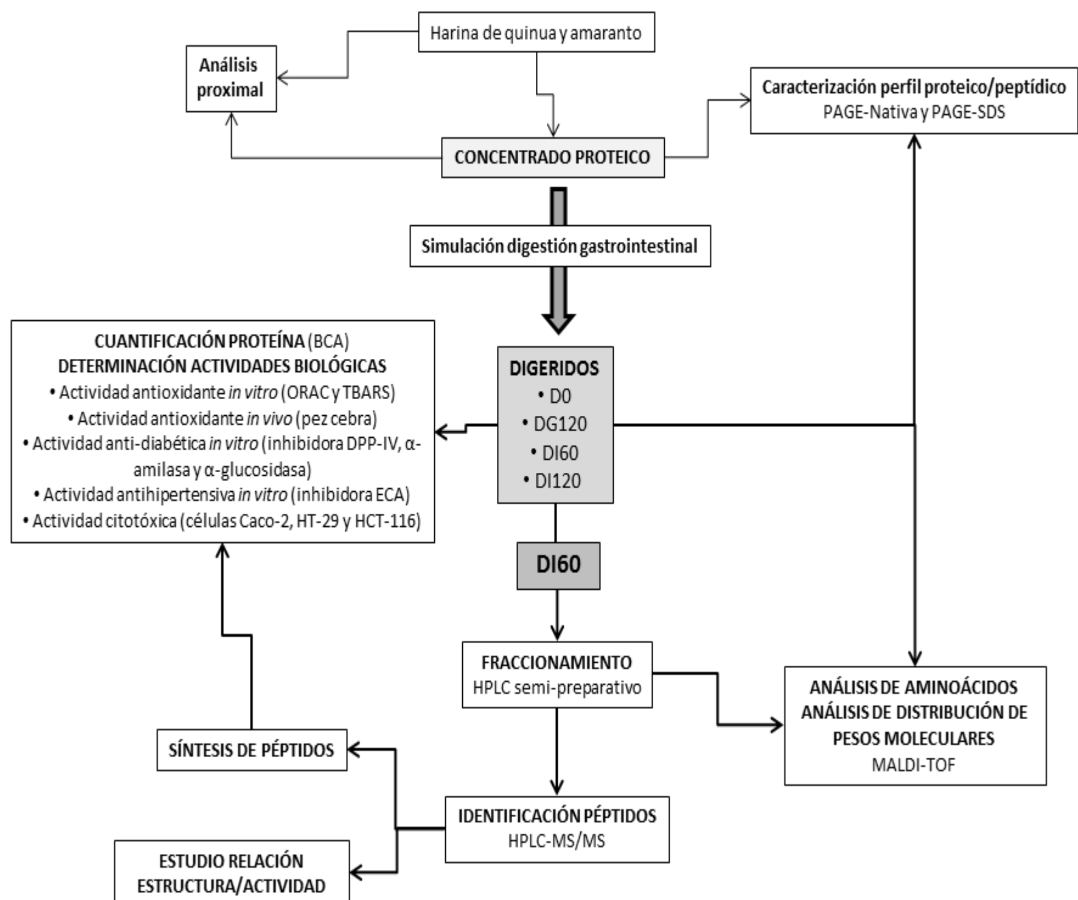
1. Caracterizar las proteínas presentes en los concentrados proteicos obtenidos a partir de granos de quinua (*Chenopodium quinoa* Willd.) y amaranto (*Amaranthus caudatus*).

2. Evaluar el comportamiento de las proteínas de quinua y amaranto tras someterse a un proceso de digestión gastrointestinal *in vitro*.

3. Evaluar la multifuncionalidad de los digeridos obtenidos durante las distintas fases del proceso digestivo.

4. Identificar los péptidos potencialmente responsables de las actividades biológicas en base a los estudios de relación estructura/actividad.

A continuación se muestra el esquema general del Plan de Trabajo:



1. INTRODUCCIÓN

El término "pseudocereales" engloba plantas dicotiledóneas como la quinua, el amaranto, el trigo sarracero, el arfonfón y el mijo, a diferencia de los cereales que son plantas monocotiledóneas. Aunque no pertenecen a la familia de las gramíneas, los pseudocereales reciben este nombre debido a sus características, propiedades y usos semejantes a los cereales. Por su composición química, los granos de estos pseudocereales son, en general, ampliamente utilizados para consumo humano en diferentes productos (Berghofer y Schoenlechner, 2002; Janssen y col., 2017).

Botánicamente, la quinua y el amaranto comparten un ancestro en común ya que pertenecen a la familia Amaranthaceae, por lo cual se encuentran estrechamente relacionadas entre sí **(Figura 1)** (Bremer y col., 2009). Estos cultivos ancestrales han

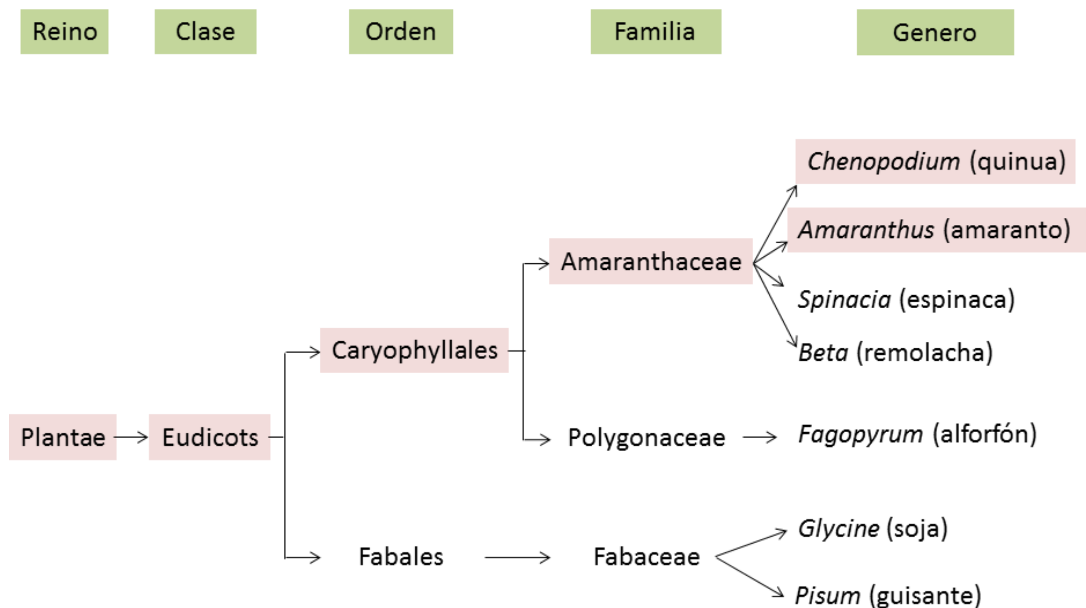


Figura 1. Filogenia de algunos pseudocereales, plantas con flores comestibles y leguminosas (Adaptada de Janssen y col., 2017).

sido usados por el hombre desde hace más de 6.000 años. Para las culturas precolombinas Mayas, Aztecas e Incas, eran cultivos de vital importancia. Sin embargo, debido a su empleo en la preparación de bebidas relacionadas con rituales religiosos,

tras la conquista por los españoles, el uso de estas plantas fue decreciendo. En los últimos años, el consumo de estos dos pseudocereales se ha incrementado nuevamente debido a su amplia variabilidad genética y alta capacidad adaptativa a diferentes tipos de suelos y hábitats agro-climáticos, además de sus características nutricionales y funcionales (Abugoch-Martínez y Añon 2003; Nascimento y col., 2014).

El género *Chenopodium* incluye alrededor de 250 especies, entre ellas la *Chenopodium quinua* Willd. y la *Ch. album* L. se presentan como las principales especies comestibles (Bhargava y col., 2005). La familia Amaranthacea tiene más de 250 especies, de las cuales únicamente tres son comestibles como grano (*Amaranthus caudatus, A. cruentus y A. hypochondriacus*) (Acosta y col., 2016). Los países de Bolivia y Perú son sus mayores productores y los principales exportadores para los mercados de Asia, Europa y Norteamérica. En general, en los últimos años se ha producido un incremento gradual en la demanda de quinua en estos mercados (Salas y col., 2017). Sin embargo su consumo en los países de altos ingresos sigue siendo bajo en comparación con los principales países productores. Así, el consumo anual (2012) de quinua en Bolivia y Perú fue de 2,37 Kg/persona y 1,15 Kg/persona, respectivamente, mientras que en USA el consumo anual fue de 0,03 Kg/persona (FAO-ALADI, 2013). En cuanto al amaranto, su consumo real y demanda aún no están bien documentados. A modo de ejemplo, México es uno de los principales países productores de amaranto y su consumo anual (2012) fue de 0,03 Kg/persona (SIAP, 2014). Una serie de productos tostados y horneados producidos a partir de las harinas de quinua y amaranto incluyen pan, galletas, fideos, pasta, panqueques, entre otros (Bhargava y col., 2006; SIAP, 2014). Además, las semillas de quinua se pueden fermentar para hacer cerveza o una bebida alcohólica ceremonial tradicional de América del Sur llamada “chicha” (FAO, 2011).

Debido a sus características tolerantes al estrés y a sus propiedades nutricionales y biológicas, se han descrito a la quinua y el amaranto como “los granos del siglo XXI” (Konishi, 2002). Por su parte, la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO) escogió a la quinua como un cultivo de gran importancia para la seguridad alimentaria, declarando 2013 el Año Internacional de la

Quinua para promover la producción, preservación y consumo de este cultivo (FAO, 2011). La quinua también fue seleccionada por la NASA (2013) para los viajes espaciales de larga duración, tomando en cuenta que por si sola puede proveer una dieta balanceada (Greg y Bubenheim 1996, 1997).

1.1 Características nutricionales de la quinua y amaranto

La **Tabla 1** muestra una comparación de los valores nutricionales de la quinua y el amaranto en relación con los del trigo y el arroz, considerados alimentos importantes en todo el mundo, tanto en dietas humanas como de animales. La superioridad de la quinua sobre estos y otros cereales (centeno, cebada, avena, entre otros) se debe a su alto contenido de proteínas, lípidos y cenizas (USDA, 2015).

El contenido de carbohidratos en la semilla de quinua es similar al del trigo y el arroz, siendo el almidón su componente principal (32% - 69%). El contenido en fibra total dietética de la quinua (7,0 - 11,7 g/100 g de materia comestible) es similar al presente en los cereales, como el trigo. De la fibra, el contenido en la parte soluble varía entre 1,3 y 6,1 g/100 g de materia comestible. Los azúcares de bajo peso molecular representan aproximadamente el 3% de las semillas de quinua, siendo maltosa, D-galactosa y D-ribosa los encontrados en mayor proporción, a diferencia de fructosa y glucosa, que se encuentran en baja concentración (Abugoch, 2009). Las semillas de quinua presentan un contenido lipídico importante (5,5 y 7,4 g/100 g de materia comestible) **(Tabla 1)**, superior al del trigo (1,7 g/100 g de materia comestible) y arroz (0,7 g/100 g de materia comestible), haciendo que la quinua sea aceptada como semilla alternativa a las semillas oleaginosas (Navruz-Varli y Sanlier, 2016). El ácido palmítico es el principal ácido graso saturado encontrado en la quinua, constituyendo el 10% de los ácidos grasos totales, mientras que los ácidos grasos insaturados: ácido oleico (19,7%-29,5%), ácido linoleico (49,0%-56,4%) y ácido alfa-linoleico (8,7%-11,7%) representan aproximadamente el 88% de la cantidad total de ácidos grasos presentes en las semillas de quinua, similar a la composición de ácidos grasos de la soja (Repo-Carrasco y col., 2003). Los ácidos grasos de las membranas celulares están bien protegidos contra daños causados por los radicales libres debido a la

presencia de la vitamina E, cuya concentración es mayor a la observada en el trigo y el arroz (Abugoch, 2009; Alvarez-Jubete, 2010).

Tabla 1. Composición nutricional de la quinua y amaranto en comparación con la de los cereales trigo y arroz (Adaptada de Becker y col., 1981; Saunders y Becker 1984; Abugoch, 2009; Vega-Gálvez y col., 2010; Hübner y Arendt, 2013; Venskutonis y Kraujalis, 2013; Ogrodowska y col., 2014; Carrillo y col., 2015; Navruz-Varli y Sanlier, 2016; Nowak y col., 2016 y Acosta y col., 2016)

Nutriente	Quinua	Amaranto	Trigo	Arroz
Energia (Kcal)	357-368	391	340	354
Proteína total[a]	13,1-16,7	13,0-19,0	11,3	6,8
Grasa total[a]	5,5-7,4	4,8-8,1	1,7	0,7
Carbohidratos[a]	59,9-74,7	71,8	63,7	79,7
Fibra[a]	7,0-11,7	3,5-5,0	12,2	0,6
Cenizas[a]	2,7-3,8	3,0-3,3	1,5	0,5
Minerales[b]				
Ca	27,5-148,7	187,0	35,0	22,0
Fe	1,4-16,7	10,0	5,0	1,4
Mg	26,0-502,0	288,0	103,0	NA
P	140,0-530,0	455,0	393,0	119,0
K	696,7-1475,0	420,0	478,0	80,0
Na	11,0-31,0	32,0	2,0	31,0
Zn	2,8-4,8	1,0	3,7	0,6
Cu	1,0-9,5	4,0	0,4	0,1
Vitaminas[b]				
Ácido ascórbico (C)	4,0-16,4	1,25-2,9	ND	ND
α-tocoferol (E)	2,6-5,4	1,9	1,4	0,7
Tiamina (B_1)	0,3-0,4		0,5	0,2
Riboflavina (B_2)	0,3-0,4	0,2	0,1	0,1
Niacina (B_3)	1,1-1,5	1,6-2,8	5,1	4,4
Piridoxina (B_6)	0,5	0,5-0,6	0,3	0,3
Folato	0,2		0,1	0,1
Aminoácidos esenciales[c]				
His	1,4-5,4	2,5	2,4	2,4
Ile	0,8-7,4	3,7	4,3	4,3
Leu	2,3-9,4	5,7	8,3	8,3
Lys	2,4-7,8	8,0	3,6	3,6
Met	0,3-9,1	4,2	2,4	2,4
Cys	0,1-2,7	4,0	2,1	2,0
Phe + Tyr	2,7-10,3	7,7	8,7	8,7
Thr	2,1-8,9	3,6	3,6	3,6
Trp	0,6-1,9	1,5	1,2	1,2
Val	0,8-6,1	4,3	6,1	6,1

[a] g/100 g materia comestible
[b] mg/100 g materia seca
[c] g/100 g proteína
NA: No disponible
ND: No detectado

Los niveles de otras vitaminas como riboflavina (B_2), piridoxina (B_6), y ácido fólico son también más altos que los contenidos en la mayoría de los cereales, como trigo, arroz, cebada y maíz. La cantidad de piridoxina y ácido fólico determinada por cada 100 g de quinua pueden satisfacer las necesidades diarias de los adultos (Abugoch, 2009). Altos niveles de vitamina C se han encontrado en las semillas de quinua, con valores que oscilan entre 4,0 mg y 16,4 mg/100 g de materia seca **(Tabla 1)**. Sin embargo, el contenido de tiamina es menor que el de la avena y la cebada (Navruz-Varli y Sanlier, 2016). Respecto a los minerales, las concentraciones encontradas en la quinua son importantes, con un alto contenido de calcio, magnesio, hierro, cobre y zinc. Además, el calcio, magnesio y potasio se encuentran en la quinua en formas biodisponibles, por lo que la ingesta de estos minerales a través de este pseudocereal se considera adecuado para mantener una dieta equilibrada (Repo-Carrasco y col., 2003; Vega-Gálvez y col., 2010).

El contenido de proteínas en las semillas de quinua oscila entre el 13,1% y el 16,7%, dependiendo de la especie. Estos valores son superiores a los encontrados en el arroz, cebada, maíz, centeno y trigo. Las proteínas de las semillas se pueden clasificar en dos grandes grupos según su función biológica: proteínas constitutivas y proteínas de almacenamiento. Las proteínas constitutivas son las responsables del mantenimiento del metabolismo celular, mientras que las de almacenamiento son proteínas producidas durante el desarrollo de la semilla y se usan tras hidrólisis durante la germinación para obtener carbono, nitrógeno y aminoácidos. Estas proteínas se caracterizan por tener, en su mayoría, pesos moleculares altos y ser poco solubles en agua (Mandal y Mandal, 2000). Las proteínas de almacenamiento fueron clasificas por Osborne de acuerdo a su solubilidad, en albúminas, solubles en agua; globulinas, solubles en concentraciones salinas altas; prolaminas, solubles en alcohol acuoso y glutelinas solubles en soluciones ácidas o alcalinas (Cunsolo y col., 2012). Las albúminas y las globulinas son las proteínas mayoritarias en la quinua, con porcentajes del 35% y 37% respectivamente, mientras que las prolaminas se encuentran presentes en bajas concentraciones (0,5-7%) (Abugoch, 2009). El principal componente de las globulinas es la globulina 11S, también conocida como chenopodina (Brinegar y

Goundan, 1993; Barrett, 2006). Además de su alta cantidad, las proteínas de quinua presentan una alta calidad debido a su patrón equilibrado de aminoácidos esenciales **(Tabla 1)** y su alto grado de digestibilidad (83%) que se establece midiendo la diferencia entre el nitrógeno absorbido y el nitrógeno ingerido (Ruales y Nair, 1993; USDA, 2015). Todos los aminoácidos esenciales para los adultos están presentes en las proteínas de la quinua, de acuerdo a los requisitos sugeridos por el comité de expertos de FAO/WHO/UNU (WHO, 2007), presentando un alto contenido en lisina (2,4 a 7,8 g/100 g de proteína), metionina (0,3-9,1 g/100 g de proteína) y treonina (2,1-8,9 g/100 g de proteína), que son los aminoácidos limitantes en los cereales convencionales, como el trigo y el maíz (Dini y col., 2005).

El amaranto ha sido considerado como una de las plantas más nutritivas del mundo, es especial por su alto contenido de proteínas, calcio, y vitamina C **(Tabla 1)** (Bressani y col., 1987b). En cuanto a los carbohidratos, estos constituyen el mayor componente de los macronutrientes presentes en la semilla de amaranto, siendo el almidón el principal componente de esta fracción (Teutonico y Knorr, 1985). En general, las semillas de amaranto contienen entre el 65% y el 75% de almidón, 4-5% de fibra total dietética y un contenido de sacarosa de dos a tres veces superior al presente en el grano de trigo. La sacarosa es el principal carbohidrato de bajo peso molecular seguido de la rafinosa, mientras que el inositol, la estaquiosa y la maltosa se encuentran en pequeñas cantidades en la mayor parte de especies estudiadas de amaranto (Becker y col., 1981). Otro componente nutritivo muy importante en las semillas del género *Amaranthus* son los lípidos, que dependiendo de la especie se pueden encontrar en concentraciones que oscilan entre el 4,8% y el 8,1% **(Tabla 1)** (Saunders y Becker, 1984). Los principales ácidos grasos encontrados en el aceite de amaranto son el ácido palmítico, ácido oleico y ácido linoleico. Estudios realizados en aceites de diferentes especies de amaranto (*A. caudatus, A. hybridus, A. cruentus y A. hypochondriacus*) mostraron porcentajes de los ácidos palmítico, esteárico, oleico y linoleico variables entre el 16,83-23,83%, 1,86-4,11%, 20,29-35,46% y 38,25-57,86%, respectivamente (Bressani y col., 1987b).

El contenido en proteína del amaranto es superior al presente en la mayoría de los cereales (maíz, arroz, trigo), variando entre un 13% y 19%, con una digestibilidad del 90% (Zheleznov y col., 1997; Acosta y col., 2016). Este contenido de proteína en la semilla está distribuido en el endospermo (35%), y el resto en la cáscara y germen (Berganza y col., 2003). El 50% del total de proteínas de la semilla corresponden a las proteínas de almacenamiento globulina y albúmina (Paredes-López y col., 1988; Soriano-Santos y Escalona-Buendia, 2015). Las globulinas constituyen la fracción proteica más abundante en aislados de proteína de amaranto, cuyo componente principal es la globulina 11S, también denominada amarantina. La fracción menos abundante corresponde a las prolaminas (1,0%-3,2%) (Quiroga y col., 2010). En cuanto a su composición, las proteínas de amaranto poseen mayor cantidad de aminoácidos azufrados que las proteínas de leguminosas, y mayor contenido de aminoácidos aromáticos e hidrofóbicos que los cereales (Gorinstein y col., 2002). Las proteínas de amaranto son ricas en lisina, con un contenido dos veces mayor (8,0 g/100g de proteína seca) al del trigo y el arroz y tres veces el del maíz **(Tabla 1)**. El balance de aminoácidos en el amaranto está cercano al requerido para una adecuada nutrición humana (Pérez-Conesa y col., 2002; FAO, 2013). De hecho, la combinación de harinas de amaranto y maíz en una proporción de 50:50 alcanza la puntuación de 100 del patrón de referencia óptimo de proteínas en la dieta (Mlakar y col., 2009).

1.2 Propiedades biológicas de la quinua y amaranto

Además de su alto valor nutritivo, la quinua ha demostrado ejercer beneficios sobre grupos de consumidores de alto riesgo como niños, ancianos, intolerantes a la lactosa y celíacos. Estos beneficios se han relacionado con su contenido en proteínas, ácidos grasos, fibra, vitaminas, minerales, y especialmente la presencia de una variedad de fitoquímicos, entre ellos saponinas, fitoesteroles, fitoecdisteroides y compuestos fenólicos que podrían ejercer efectos sobre la salud a nivel metabólico, cardiovascular y gastrointestinal (Navruz-Varli y Sanlier, 2016; Vilcacundo y Hernández-Ledesma, 2017).

Se han descrito actividades biológicas para las saponinas presentes en la capa exterior de las semillas de quinua como las actividades anti-fúngica, anti-viral, anti-cancerígena, hipoglucémica, anti-trombótica, diurética y anti-inflamatoria (Graf y col., 2015). Los fitoesteroles por su parte han demostrado tener actividad antioxidante, anti-inflamatoria, anti-cancerosa y capacidad reductora de los niveles de colesterol (Ho y Pal, 2005; Ryan y col., 2007). Sin embargo, aunque muchos componentes de la quinua han sido descritos como beneficiosos para la salud humana, hasta la fecha, la evidencia de estos efectos, tanto en animales como en humanos, sigue siendo limitada. En un estudio sobre los efectos de este pseudocereal en el perfil lipídico y los niveles de glucosa en ratas macho Wistar, se demostró la capacidad de las semillas de quinua para reducir los niveles de colesterol total, lipoproteínas de baja densidad (LDL), triglicéridos y glucosa en suero, tras ser alimentadas con una dieta enriquecida con fructosa (Pasko y col., 2010). En otro estudio, la administración de un suplemento de quinua en ratas con estrés oxidativo inducido, redujo los niveles de malondialdehído en plasma, aumentando la actividad de las enzimas antioxidantes (Pasko y col., 2010). Estos resultados indican que las semillas de quinua podrían proteger al organismo de los efectos del estrés oxidativo debido al incremento de la capacidad antioxidante y la reducción de la peroxidación lipídica en plasma y diferentes tejidos. Foucaul y col. (2011) investigaron el potencial de la quinua para prevenir la obesidad en ratones. La administración de un extracto de quinua enriquecido con 20-hidroxiecdisona (20HE) durante 3 semanas a ratones con una dieta rica en grasas provocó la reducción del desarrollo del tejido adiposo sin cambios en la ganancia del peso corporal. El efecto sobre el tejido adiposo se asoció con la regulación de los genes implicados en el almacenamiento de lípidos (Foucaul y col., 2011). La administración, dos veces al día durante 15 días, de 100 g de quinua (alimentos de quinua para bebés) a niños de 50 a 65 meses de edad, pertenecientes a familias de bajos ingresos de Ecuador, aumentó significativamente los niveles del factor de crecimiento similar a la insulina (IGF-1) en plasma, en relación al grupo control. Esto indica que los alimentos de quinua para bebés proporcionaron suficiente proteína y otros elementos nutricionales capaces de prevenir la desnutrición en la población infantil (Ruales y col., 2002). Además, la

suplementación en la dieta con quinua ha demostrado que puede prevenir desordenes cardiovasculares en personas sanas (Farinazzi y col., 2012) y es capaz de modular parámetros metabólicos en el sobrepeso post-menopáusico de las mujeres (De Carvalho y col., 2014). La quinua también fue administrada a pacientes celíacos, con el fin de evaluar la seguridad de su consumo como alimento libre de gluten, y siendo una alternativa a los cereales tradicionales (Zevallos y col., 2014). Ese estudio, después de 6 semanas de consumo de 50 g de quinua al día, demostró una mejora de los parámetros gastrointestinales así como la disminución de los niveles de colesterol total, LDL, lipoproteínas de alta densidad (HDL) y triglicéridos.

Además de su valor nutricional, el grano de amaranto contiene varios compuestos bioactivos, que incluyen flavonoides, ácidos fenólicos, antocianinas, taninos, fitoesteroles y escualeno (Venskutonis y Kraujalis, 2013). Lin y col. (2005) demostraron que el extracto acuoso de la hoja de *A. spinosus* debe sus propiedades inmunoestimulantes a una proteína de 313 kDa llamada GF1. Esta proteína tiene la capacidad de estimular la activación de linfocitos B, con la consecuente proliferación de linfocitos T *in vitro* (Lin y col., 2005). En cuanto a su potencial antioxidante, un estudio comparó el amaranto con granos de cereales, y se observó que el amaranto tiene una mayor capacidad de neutralización de radicales 1,1-difenil-2-picrilhidracil (22,6 mg ácido gálico equivalente (GAE)/g de muestra) que los cereales cebada, trigo, arroz, mijo japonés y trigo sarreceno (2,5 a 17,7 mg GAE/g de muestra) (Asao y Watanabe, 2010). Por otro lado, se ha comprobado tanto en estudios *in vitro* como *in vivo*, que el grano y el extracto acuoso del grano de *A. hypochondriacus* L. inhiben la producción de inmunoglobulinas IgE antígeno-específica implicadas en respuestas de hipersensibilidad y alergias, a través de la inducción de citoquinas proinflamatorias (Th1). Los estudios *in vitro* demostraron que la fracción soluble en agua del grano de amaranto promovió el desarrollo del fenotipo de células T tipo 1 colaboradoras (Th1) y suprimió la producción de IgE, mientras que el estudio *in vivo* mostró el efecto del amaranto administrado oralmente sobre la concentración de IgE en suero que refleja la respuesta inmune durante la enfermedad alérgica. Se observó una disminución significativa ($p < 0,05$) de la IgE en la sangre de los ratones (Hibi y col., 2003). Por

tanto, se ha sugerido al amaranto como una herramienta útil en el tratamiento de enfermedades alérgicas (Guerra-Matias y Arêas, 2005).

También se ha recomendado el consumo de amaranto en la dieta es recomendado en pacientes con enfermedades cardiovasculares (Martirosyan y col., 2007) debido al efecto demostrado para esta planta como modulador del perfil lipídico plasmático y de los niveles de colesterol sérico, en ensayos en animales y humanos, (Caselato-Sousa y Amaya-Farfán, 2012). En la medicina tradicional china, el amaranto es empleado comúnmente para tratar la diabetes. Un estudio evaluó la digestibilidad de un producto extruido a partir de amaranto. Se observó el comportamiento de la digestión en las respuestas glucémicas e insulinémicas de 11 mujeres a las cuales se les subministró este producto, en comparación con las respuestas a un alimento de referencia (pan blanco). El índice glucémico y la curva de insulina de estas mujeres que consumieron el producto de amaranto indicaron la capacidad de este pseudocerel para estimular la producción de insulina en relación al alimento control (Guerra-Matias y Arêas, 2005). También han sido descritos efectos inmunomoduladores en amaranto. Se ha observado que la ingesta de alimentos preparados con amaranto no provoca reacciones alérgicas en la mucosa intestinal, por lo que el consumo de estos alimentos se recomienda en pacientes celíacos (Guerra-Matias y Arêas, 2005) e hipercolesterolémicos (Czerwiński y col., 2004). De manera general, los estudios de las propiedades bioquímicas y farmacológicas del amaranto han permitido considerar a esta planta como un alimento funcional (Silva-Sánchez y col., 2008).

1.3 Péptidos con actividad biológica

En la actualidad, existe un creciente interés por los alimentos funcionales, suplementos dietéticos y preparaciones farmacéuticas que contienen compuestos bioactivos. Un número considerable de estudios se han enfocado en la evaluación de las actividades biológicas de péptidos y el posterior desarrollo de alimentos funcionales que los contengan. Así como la mayoría de estos estudios se han centrado en proteínas de origen animal, en los últimos años el interés por péptidos procedentes de proteínas vegetales se ha incrementado notablemente (Rizzello y Col., 2016). Los

péptidos bioactivos pueden generarse a partir de una proteína precursora tras la acción de las enzimas digestivas durante la digestión gastrointestinal, durante el procesado de los alimentos (maduración, fermentación, cocción o hidrólisis *in vitro* con enzimas proteolíticas), o el almacenamiento de los alimentos (Meisel, 1998; Vioque y col., 2000b; Carrasco-Castilla y col., 2012). Estos péptidos contienen entre 3 y 20 aminoácidos, pero en algunos casos el tamaño es mayor (Shahidi y Zhong, 2008). Debido a las actividades que estos péptidos pueden ejercer sobre diferentes sistemas del organismo, han sido considerados como componentes prometedores para el desarrollo de nuevos alimentos funcionales (Gobbetti y col., 2007).

La evaluación del potencial de las proteínas vegetales como fuente de péptidos bioactivos es de gran interés, debido a la sostenibilidad de dichas proteínas con respecto a las proteínas de origen animal y a la mayor preocupación por los consumidores por dietas saludables y equilibradas. Diferentes estudios indican que los cereales y las leguminosas son fuente de proteínas y péptidos con actividad biológica (García y col., 2013; Malaguti y col., 2014), pero también se han encontrado péptidos bioactivos provenientes de otras fuentes vegetales (algas, hongos comestibles, ajo, cúrcuma, sésamo, cacahuete, alfalfa, espinaca, semillas de cáñamo, tubérculos, granos de cacao y otros), tras procesos de fermentación e hidrólisis enzimática (García y col., 2013). Dichos estudios describen los efectos funcionales de péptidos bioactivos procedentes de proteínas vegetales, incluyendo actividad antioxidante, antiproliferativa, antihipertensiva y anti-inflamatoria, entre otras (Rizzello y col., 2016). A continuación, se irán detallando las actividades más destacadas de péptidos procedentes de proteínas vegetales.

1.3.1. Actividad antioxidante

Durante el metabolismo celular, el oxígeno es parcialmente reducido a especies reactivas, siendo las procedentes del oxígeno (ROS) las más abundantes, seguidas de las de nitrógeno (RNS) y de cloro (RCS). Todas ellas son, en cierta medida, beneficiosas a nivel fisiológico, ya que apoyan al sistema inmune y actúan como segundos mensajeros en una gran cantidad de procesos celulares, como la

proliferación, el ciclo celular, la muerte celular y la transducción de señales (Martindale y Holbrook, 2002). Sin embargo, su alta reactividad puede desencadenar daños mediante ataques nucleofílicos, provocando peroxidación lipídica, oxidación proteica y, lo más relevante, alteraciones genéticas como daño de ADN, mutaciones, cambios epigenéticos e inestabilidad genómica (Khan y col., 2008).

En el organismo humano, la producción de las especies reactivas puede controlarse mediante los sistemas de defensa antioxidante, tanto de tipo enzimático como no enzimático. No obstante, cuando se produce un desequilibrio debido a alteraciones en dichos sistemas de defensa o una producción excesiva de especies reactivas se produce un estado denominado “estrés oxidativo”. Este estado y sus posteriores daños sobre los componentes celulares se asocian con numerosas enfermedades degenerativas graves, como los desórdenes cardiovasculares y neurodegenerativos, la diabetes, el síndrome metabólico, las cataratas, algunos tipos de cáncer y el propio envejecimiento (Bray, 2000) **(Figura 2)**. Además, las reacciones de oxidación participan en el origen de múltiples procesos relacionados con la pérdida de calidad en los alimentos, como la rancidez de las grasas, los cambios de color y el deterioro nutricional del alimento, disminuyendo su caducidad (Shahidi y col., 1992).

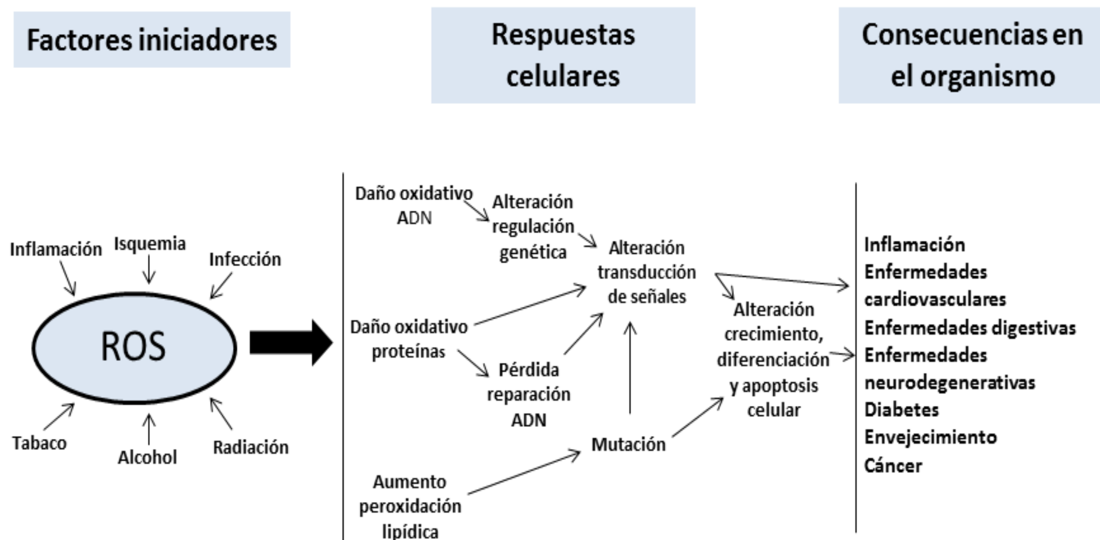


Figura 2. Representación esquemática de la inducción del estado de estrés oxidativo y sus efectos patofisiológicos (Adaptada de Fernández-Tomé, 2016).

El tracto gastrointestinal representa un sistema de interfaz complejo entre el cuerpo y el medio ambiente externo. Para evitar el paso de moléculas peligrosas, patógenas y endotoxinas, el organismo ha desarrollado varios mecanismos protectores a este nivel gastrointestinal, como la modulación del pH, el peristaltismo, la capa de gel mucosal y el tejido linfoide asociado al intestino. A pesar de esta barrera protectora, la mucosa intestinal está continuamente expuesta a una alta concentración de ROS procedente de compuestos endógenos en la superficie luminal, así como de fuentes exógenas y de la propia microbiota intestinal (Couto y col., 2012; Graham-Espey, 2013). Por lo tanto, en numerosos estudios se ha demostrado la influencia del estrés oxidativo en el desarrollo de múltiples trastornos gastrointestinales, incluyendo el cáncer colorrectal (Kim y col., 2012; Bhattacharyya y col., 2014). Las estrategias actuales de intervención farmacológica están principalmente basadas en el uso de fármacos corticosteroides y otros agentes inmunosupresores. Sin embargo, estos fármacos presentan varios efectos adversos tras su administración prolongada, como alteraciones gastrointestinales, anemia, hepatotoxicidad, nefrotoxicidad y reacciones de hipersensibilidad (Rutgeerts y col., 2009). Además, el uso crónico de estos fármacos se ha asociado a una menor respuesta farmacológica debido a una falta de efectividad de los mismos (Barnes y Adcock, 2009). Por ello, y con el objeto de evitar inconvenientes, numerosas investigaciones se han centrado en la búsqueda e identificación de nuevos agentes de origen natural con potencial efecto antioxidante (Moura y col., 2015).

Se han descrito varios métodos para evaluar la capacidad antioxidante de un compuesto. Inicialmente, la mayoría de las investigaciones se centraron en distintos métodos químicos *in vitro*, basados en la complejidad de las reacciones de oxidación que ocurren en los sistemas biológicos. Estos métodos químicos pueden clasificarse en ensayos basados en la transferencia de un átomo de hidrógeno, entre los que destaca el ensayo “oxygen radical absorbanse capacity (ORAC)” y el ensayo “total radical trapping antioxidant parameter (TRAP); y los basados en la trasferencia de electrones, como el ensayo “Trolox equivalent antioxidant capacity (TEAC)”, el ensayo “ferric ion reducing antioxidant parameter (FRAP)” y el ensayo “radical scavenging capacity assay (DPPH)”, entre otros (Huang y col., 2005). El ensayo ORAC, a diferencia de la simple

medición del contenido de antioxidantes presentes en un alimento, mide la actividad o capacidad global que tienen todos los antioxidantes presentes en una muestra para neutralizar los radicales peroxilo. Estos últimos son especies reactivas comparables y por tanto relevantes a aquellos ROS biológicamente generados en el organismo. Como tal, el valor ORAC constituye el índice *in vitro* más reconocido hasta el momento para definir el potencial aporte que podría suponer el consumo de un alimento a la capacidad antioxidante de nuestro organismo (Huang y col., 2005; Prior y col., 2005). De la misma manera, los modelos celulares son considerados una herramienta útil para validar alimentos ricos en antioxidantes naturales, ya que proporcionan una valiosa información sobre la eficacia protectora de sustancias bioactivas puras y los diferentes mecanismos de acción (O´Brien y col., 2000; Glei y col., 2003; Lui y Finley, 2005). La valoración del efecto protector proporcionado por la muestra se realiza mediante la evaluación de diferentes parámetros o biomarcadores que indican el daño causado por el agente oxidante. Así, se evalúan cambios en la: tasa de muerte celular, la actividad de los sistemas enzimáticos antioxidantes y/o niveles del glutatión reducido (GSH), formación de productos de peroxidación lipídica (malondialdehido, isoprostanos), generación intracelular de ROS, oxidación proteica (formación de grupos carbonilo) y oxidación del ADN (Nardini y col., 1998; Erba y col., 1999; Aherne y O´Brien, 2000; O´Brien y col., 2000; Miranda-Rottmann y col., 2002; Sestili y col., 2002; Feng y col., 2002). Dentro de los modelos experimentales *in vivo*, el modelo de pez cebra está siendo utilizado para la evaluación de la actividad antioxidante. En sus larvas se ha estudiado el efecto inhibitorio de la peroxidación lipídica, constituyendo este sistema una herramienta previa a los modelos animales superiores y a los ensayos clínicos (Carrillo y col., 2016).

Los péptidos antioxidantes contienen entre 5 y 16 residuos de aminoácidos. Su actividad antioxidante puede estar relacionada con la quelación iónica, la eliminación de radicales libres y la inhibición de la peroxidación lipídica (Dávalos y col., 2005; Kim y col., 2007). Se han llevado a cabo estudios con péptidos antioxidantes obtenidos a partir de proteínas vegetales. Así por ejemplo, el tratamiento del aislado de proteína de soja con las enzimas pepsina, papaína, quimotripsina, Alcalasa®, Protamex®, y

Flavorzima® empleadas por separado, ocasionó la obtención de distintos hidrolizados cuya actividad antioxidante, utilizando el método de las sustancias reactivas al ácido tiobarbitúrico TBARS, fue del 28%-65% (Liu y col., 2005). Estos investigadores demostraron además que la leche de soja fermentada inoculada con 5% de kéfir (p/v) e incubada a 20ºC durante 32 horas, poseía actividad antimutagénica (45,7-68,8%) evaluada mediante el test de Ames y actividad antioxidante determinada mediante la inhibición de la peroxidación lipídica (45%-65%). Otro estudio realizado con la zeína (proteína obtenida del maíz), evaluó el potencial antioxidante de hidrolizados obtenidos a partir de la digestión con Alcalasa®, y posterior tratamiento con pepsina/pancreatina. Los resultados mostraron que la digestión *in vitro* del hidrolizado de zeína contenía hasta un 16,5% de aminoácidos libres y una elevada proporción de péptidos de bajo peso molecular (< 500 Da). Las fracciones peptídicas ricas en di-, tri- y tetra-péptidos (1-8 mg/mL de proteína) mostraron una actividad antioxidante comparable e incluso mayor a la producida por el ácido ascórbico o el butilhidroxianisol BHA, a una concentratración de 0,1 mg/mL (Zhu y col., 2008). Sin embargo, la secuencia de los péptidos responsables de la actividad no fue descrita. Zhang y col. (2011) purificaron un péptido con actividad antioxidante obtenido a partir de un hidrolizado de proteína de garbanzo digerido con Alcalasa®. La secuencia de aminoácidos del péptido se identificó como NRYHE, con un peso molecular de 717,37 Da. Este péptido presentó capacidad de captación de radicales libres 2,2-Difenil-1-Picrihidrazilo (DPPH), hidroxilo y superóxidos. Además la inhibición de la peroxidación lipídica resultó ser mayor (82,93%) a la producida por el α-tocoferol (vitamina E) (75,69%), cuando se ensayaban a la misma concentración de 50 μg/mL. Chuang-He y col. (2009) demostraron que los productos de la hidrólisis del aislado proteico de trigo sarraceno obtenidos con Alcalasa® presentaron excelente actividad antioxidante mediada por la captación de radicales libres, efectos reductores e inhibición de la peroxidación del ácido linoléico. Ma y col. (2010) hidrolizaron el mismo aislado proteico empleando pepsina y pancreatina, encontrando que el hidrolizado obtenido tras 2 horas de digestión con pancreatina presentó mayor actividad antioxidante determinada por la capacidad de captación de radicales 2,2´-azinobis (3-etilbenzotiazolin)-6-sulfónico (ABTS) y radicales

hidroxilo. Posteriormente, este hidrolizado fue separado en seis fracciones, siendo las fracciones IV (456 Da) y VI (362 Da) las que mostraron la mayor capacidad de captación de radicales ABTS (un promedio de 24,5% más activas en comparación con el digerido completo). Finalmente, se identificaron las secuencias de los péptidos potencialmente responsables de la actividad, que correspondieron a WPL, VPW Y VFPW. En un estudio llevado a cabo por Zhang y col., (2010), la proteína desengrasada del endospermo de arroz fue hidrolizada empleando diferentes proteasas (Alcalasa®, Neutrasa®, Flavorzima®, quimotripsina y papaína). El hidrolizado enzimático obtenido con Neutrasa® presentó los valores más altos de actividad antioxidante bien determinada por la captación de radicales libres DPPH (85,86%), hidroxilo (82,93%) y superóxidos (75,69%) y la inhibición de la autooxidación del ácido linoleico (80,09 %). Además se identificaron dos péptidos, con las secuencias FRDEHKK (959,5 Da) y KHDRGDEF (1002,5 Da), que mostraron una fuerte actividad antioxidante (Zhang y col., 2010). El péptido FRDEHKK fue sintetizado y se determinó que la actividad antioxidante de este péptido en un sistema modelo de ácido linoléico fue más eficaz que el α-tocoferol (86,59%). Además, el péptido a una concentración de 80 µg/mL, aumentó la viablidad de células MRC-5 (74,38%) y RAW264.7 (78,39%) inducidas por hidroperóxido de tert-butilo (*t*-BOOH).

Entre los péptidos bioactivos con potencial antioxidante presentes en la soja destaca el péptido llamado lunasina, compuesto por 43 aminoácidos y presente en la fracción 2S albúmina de este leguminosa (De Mejía y de Lumen, 2006). Se ha demostrado la actividad beneficiosa de este péptido al disminuir los marcadores de estrés inflamatorio y oxidativo inducido por lipopolisacárido (LPS) en macrófagos RAW 264.7 (Vernaza y col., 2012). De forma similar, se observaron efectos protectores frente al estrés oxidativo inducido mediante los agentes químicos H_2O_2 y *t*-BOOH en células intestinales Caco-2 diferenciadas (García-Nebot y col., 2014).

1.3.2 Actividad antidiabética

La diabetes mellitus tipo 2 (DMT2) es un trastorno metabólico crónico considerado como uno de los principales problemas de salud, debido a su creciente

incidencia y tasa de prevalencia (WHO, 2016). La DMT2 se caracteriza por la resistencia a la insulina y la falta relativa de la misma. A su vez, la resistencia a la insulina conduce a un aumento en los niveles de glucosa en sangre con el resultado de una hiperglucemia postprandial (Robertson, 1995; De Fronzo, 1999). La DMT2 afecta el metabolismo de los carbohidratos, proteínas y grasas, y es la principal causa de trastornos cardiovasculares y neurológicos, ceguera, insuficiencia renal y amputación de los miembros inferiores (Jao y col., 2015). Actualmente, se utilizan varias estrategias en la prevención y manejo de la DMT2 como son los cambios en la dieta eliminando los alimentos procesados e incrementando la ingesta de frutas y verduras, el ejercicio físico regular y el uso de diferentes fármacos antidiabéticos (Patil y col., 2015). Sin embargo, debido a los efectos adversos de los agentes convencionales antihiperglucémicos, últimamente se ha potenciado la investigación sobre compuestos de origen natural que sean seguros y puedan prevenir la progresión de la DMT2 y sus respectivos trastornos (Majumdar y Inzucchi, 2013). Una de las nuevas estrategias se basa en la inhibición de la enzima dipeptidil peptidasa IV (DPP-IV), responsable de la degradación e inactivación de las hormonas incretinas (péptido similar al glucagón tipo 1 (GLP-1) y péptido insulinotrópico dependiente de la glucosa (GIP)). Estas hormonas son secretadas en respuesta a la presencia de nutrientes en el lumen intestinal **(Figura 2)**, y actúan estimulando la secreción de insulina (glucosa-dependiente) por parte de las células beta pancreáticas (Holst y Deacon, 2004; Mentlein, 2005). La administración intravenosa de GLP-1 en individuos diabéticos regulariza los niveles de glucosa en sangre (Nauck y col., 1993). Sin embargo, los efectos de esta hormona incretina son de corta duración, debido a la rápida degradación e inactivación de GLP-1 en plasma sanguíneo por la DPP-IV (Nauck y col., 1996).

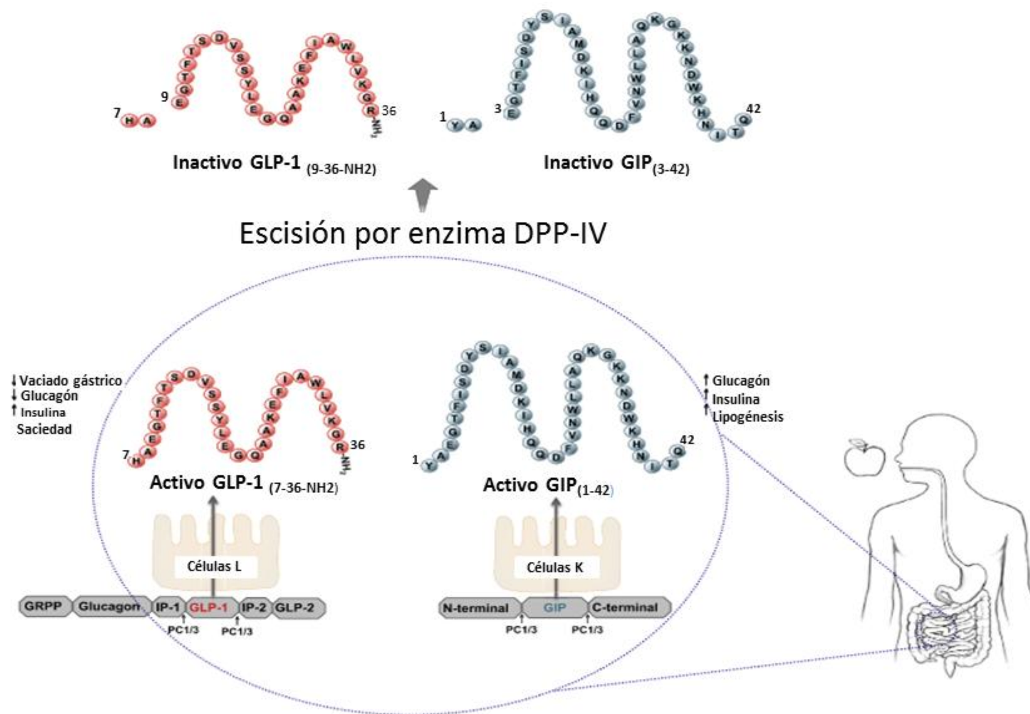


Figura 2. Representación esquemática de la generación, después de la ingesta de alimentos, de las hormonas incretinas GLP-1 y GIP, y su división por acción de la enzima DPP-IV (Tomada de Lacroix y Chan, 2016).

Ensayos en modelos animales y en seres humanos han demostrado que la inhibición de la DPP-IV aumenta el promedio de vida del GLP-1 circulante, reduciendo los niveles de glucosa plasmática y la tolerancia mejorada de glucosa (Deacon y col., 1998; Deacon y col., 2000; Mitani y col., 2002). Por lo tanto, inhibidores de la enzima DPP-IV han surgido en los últimos años como una nueva clase de agentes orales para el tratamiento de la DMT2 (Hunziker y col., 2005; McIntosh y col., 2005). Además, el bloqueo de la acción de las enzimas responsables de la hidrólisis de carbohidratos, tales como la α-amilasa y α-glucosidasa, se presenta como otra alternativa en el control de la homeostasis de la glucosa en pacientes diabéticos (Johnson y col., 2011). Se sabe que los inhibidores de la α-amilasa salivar y pancreática bloquean la hidrólisis de los almidones complejos a oligosacáridos, evitando que la glucosa presente en el almidón pueda ser absorbida por el cuerpo humano (Richardson, 1991). Además, los inhibidores de la α-glucosidasa bloquean la hidrólisis de di-, tri-, y oligasacáridos a glucosa en el intestino delgado. Por lo tanto, la inhibición de ambas enzimas disminuye

la velocidad de digestión de los carbohidratos y, como resultado, se disminuye el transporte y la absorción de glucosa hacia la circulación sistémica (El Abed y col., 2017).

Un gran número de péptidos de pequeño tamaño derivados de proteínas animales y vegetales se han reconocido como inhibidores de la DPP-IV (Jao y col., 2015; Mojica y col. 2015; Nongonierma y FitzGerald, 2015). Se ha demostrado que los péptidos derivados de las proteínas de frijol (negro, pinto, rojo, azul marino), cáñamo, arroz marrón y soja tras un proceso de digestión gastrointestinal *in vitro* poseen actividad inhibidora de la DPP-IV. Así mismo, las proteínas de las variedades de frijol pinto durango y negro 8025 presentaron actividad anti-hiperglucémica vía inhibición de la DPP-IV, tras un proceso de hidrólisis con Alcalasa® y bromelaína (Oseguera-Toledo y col., 2015). A pesar de que la actividad inhibidora de la DPP-IV ha sido demostrada en numerosos estudios *in vitro,* la información disponible en la literatura científica sobre la actividad *in vivo* de estos inhibidores naturales es todavía bastante escasa. La administración ileal a ratas de un hidrolizado de zeína producido con papaína antes del test de tolerancia a la glucosa intraperitoneal, demostró aumentar la secreción de insulina 2,4 veces a los 15 minutos y disminuir los niveles de glucosa en sangre, en comparación con el grupo control (Mochida y col., 2010). Además, la administración del hidrolizado de zeína provocó un aumento en las concentraciones de GLP-1 total y GLP-1 activo, relacionándose el incremento de esta última hormona con los cambios en los niveles de insulina y glucosa. Ishikawa y col. (2015) demostraron que los animales que recibieron oralmente un hidrolizado de proteína del salvado de arroz, tuvieron una respuesta glucémica atenuada y un aumento en la concentración plasmática de GLP-1 durante un test de tolerancia a la glucosa intraperitoneal. Además, se encontró que la actividad de la DPP-IV en plasma se redujo después de la administración ileal de los hidrolizados. En otro estudio realizado con lisozima hidrolizada con Alcalasa®, se demostró la reducción de la actividad de la DPP-IV en ratas diabéticas (Wang y col., 2012). A estos animales se les administró vía oral el hidrolizado, mostrando después de 90 min una reducción del 25% de la actividad de la DPP-IV en suero sanguíneo. Se observó también un incremento de 1,4 veces en la concentración sérica de GLP-1. Sin

embargo, tras 15 semanas de tratamiento con el hidrolizado o el inhibidor sintético vildagliptina, no se observó una mejora significativa en parámetros metabólicos, como la glucosa en sangre o insulina en suero, sin determinarse el mecanismo que explique la falta del efecto a largo plazo (Wang y col., 2012).

En el caso de los inhibidores naturales de la α-amilasa y α-glucosidasa, los estudios son más limitados, aunque recientemente Mojica y De Mejía (2016), determinaron el potencial biológico de los péptidos liberados tras la acción proteolítica de diversas enzimas sobre aislados de proteína del frijol negro. Alcalasa® fue la enzima seleccionada para generar péptidos antidiabéticos cuyos valores de inhibición para la α-amilasa y α-glucosidasa fueron de 53,4% y 66,1%, respectivamente. El péptido TTGGKGGK presentó un mayor potencial inhibidor de la α-glucosidasa, y los péptidos AKSPLF y WEVM presentaron capacidad inhibitoria de la α-amilasa. Oseguera-Toledo y col. (2015) trabajaron con aislados protéicos de frijoles pinto durango y negro 8025, los mismos que fueron hidrolizados con Alcalasa® y bromelaína y separados en cinco fracciones peptídicas. La fracción menor a 1 kDa obtenida a partir del hidrolizado con bromelaína mostró la mayor inhibión de la α-amilasa (49%), y la fracción procedente del hidrolizado con Alcalasa® inhibió tanto la α-glucosidasa (76%) como la DPP-IV (55%). Los péptidos LLSL, QQEG y NEGEAH se encontraron presentes en las fracciones más potentes.

1.3.3 Actividad antiproliferativa

El cáncer es una de las principales causas de mortalidad y discapacidad en todo el mundo, responsable de aproximadamente el 13% de las muertes (Ferlay y col., 2010). En las últimas décadas, se ha avanzado en nuevos tratamientos contra esta enfermedad, como como quimioterapia, cirugía y radiación. Sin embargo, estas terapias son muy costosas e incluyen mediacamentos con numerosos efectos secundarios. Por lo tanto, hay una clara necesidad de buscar y desarrollar estrategias más baratas y más efectivas para maximizar las tasas de curación con toxicidad mínima. Además, se ha intensificado la búsqueda de nuevas alternativas destinadas a prevenir el tumor, reduciendo así las las altas tasas de mortalidad y los costos elevados

de las terapias. Se ha estimado que solo un 5-10% de todos los casos de cáncer se deben a defectos genéticos, mientras que el 90-95% restantes se atribuyen a factores ambientales y aquellos relacionados con el estilo de vida (Anand y col., 2008). Evidencias epidemiológicas han demostrado que las modificaciones de los factores nutricionales y los patrones de consumo de alimentos pueden potencialmente prevenir hasta el 35% de los casos de cáncer (Marmot y col., 2007), aunque el porcentaje real depende del componente dietético y el tipo específico de cáncer (Davis y Milner, 2007).

La quimioterapia se ha definido como “el uso de sustancias químicas, naturales o sintéticas que bloquean, retrasan o revierten el proceso de carcinogénesis”. Entre los agentes quimiopreventivos, se ha demostrado que aquellos presentes en los alimentos pueden tener actividad antiproliferativa reduciendo el riesgo de cáncer e, incluso, sensibilizando a las células tumorales frente a otras terapias anti-cancerígenas (Béliveau y Gingras, 2007). Una amplia investigación ha revelado que una dieta incluyendo un alto consumo de frutas, verduras y granos tiene el potencial para prevenir el cáncer. Las sustancias bioactivas contenidas en estas especies de plantas, incluyendo nutrientes esenciales, ácidos grasos poliinsaturados y fitoquímicos, son los responsables de los efectos preventivos contra el cáncer, y por lo tanto, han sido extensamente estudiados (Anand y col., 2008). Sin embargo, los compuestos bioactivos de los alimentos no son limitados únicamente a las plantas, ya que los productos alimenticios provenientes de animales y hongos tambien pueden contener compuestos con propiedades anticancerígenas. Recientemente, se ha descrito el papel crucial que desempeña la microbiota intestinal en la liberación de compuestos con efectos pro y anticancerígenos (Milner, 2014). Por tanto, el conocimiento sobre el efecto de los componenetes dietéticos en la salud es fundamental en la quimioprevención a través de un régimen alimenticio. Los polifenoles son uno de los grupos de compuestos alimenticios más estudiados por sus propiedades quimiopreventivas, destacando algunos, como las catequinas, la quercetina, la curcumina y el resveratrol (Shay y col., 2015; Bimonte y col., 2016; Lewandowska y col., 2016) En los últimos años, las proteínas y sus derivados peptídicos se han descrito también como agentes quimiopreventivos (Hernández-Ledesma y Chia-Chien

2017). Estos compuestos, al presentar gran afinidad y especificidad por ciertas dianas celulares, facilidad de penetración en los tejidos, así como bajo coste, toxicidad y efectos adversos demostrados poseen una ventaja competitiva respecto a otros tipos de moléculas quimioterapéuticas (Bhutia y Maiti, 2008). Varios estudios han demostrado el efecto quimiopreventivo de los péptidos bioactivos en cultivos de células cancerosas, y también en modelos animales a través de varios mecanismos moleculares incluyendo inducción de la apoptosis, bloqueo del ciclo celular, daño a la membrana celular, inhibición de la adhesión celular, modulación de la respuesta inmune e inhibición de la señalización intracelular (**Figura 3**) (Kim y col., 2000; Mader y col., 2005; Wang y col., 2008b; Xue y col., 2009; Barrios y Añon, 2010; Li y col., 2013; Umayaparvathi y col., 2014; Pan y col., 2016). Sin embargo, el mecanismo de acción anti-cancerígena de los hidrolizados proteicos o de los péptidos presentes en dichos hidrolizados siguen sin haberse elucidado completamente, aunque se ha descrito el papel esencial que juegan algunos aminoácidos, como prolina, leucina, glicina, alanina, lisina, arginina, serina, ácido glutámico, treonina y tirosina (Vital y col., 2014; Hung y col., 2014; Chi y col., 2015; Wang y Zhang 2017).

Se han aislado péptidos con actividad anticancerígena a partir de hidrolizados de proteínas vegetales, como el arroz, soja, algas marinas y otros. Kannan y col. (2010) identificaron el péptido de secuencia EQRPR en las proteínas del salvado de arroz digerido con la enzima Alcalasa®. Dicho péptido demostró su actividad antiproliferativa frente a diferentes líneas celulares de cáncer de colon, mama e hígado a una concentración de 600-700 µg/mL.

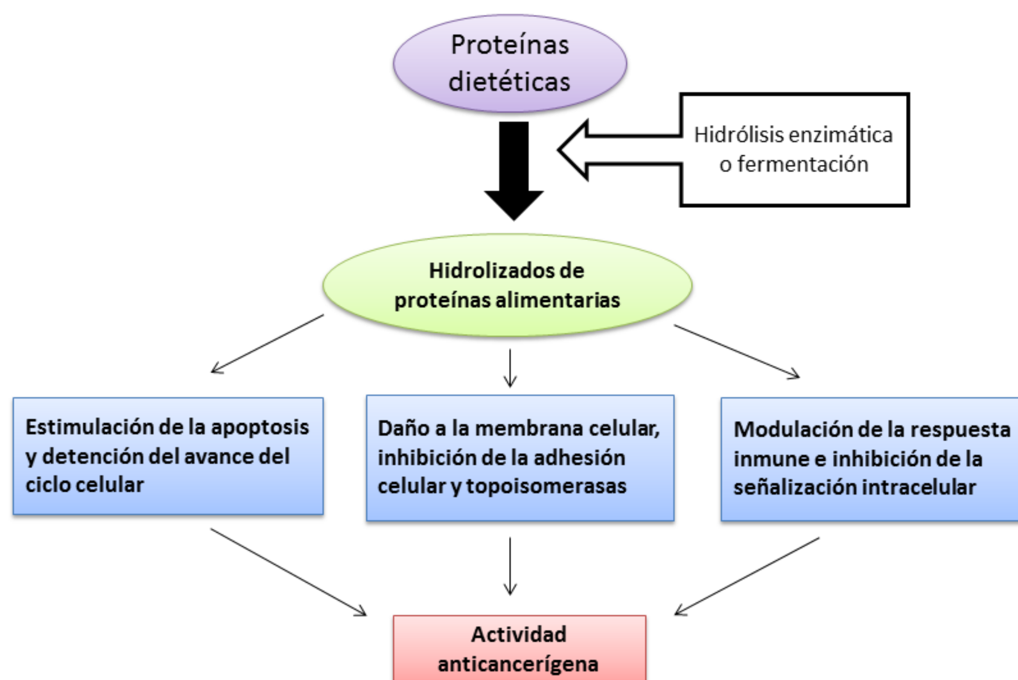


Figura 3. Mecanismos de acción de hidrolizados proteicos o péptidos con actividad anticancerígena derivados de los alimentos (Tomada de Chalamaiah y col., 2017).

El péptido con la secuencia XMLPSYSPY fue purificado y aislado de proteínas desengrasadas de la soja hidrolizadas con termolisina. Su actividad anticancerígena se ensayó midiendo la citotoxicidad *in vitro* en macrófagos de ratón P388D1. El péptido mostró una alta citotoxicidad (IC_{50} = 0,16 mg/mL) afectando el desarrollo del ciclo celular en la fase G2/M (Kim y col., 2000).

Se ha demostrado que los péptidos derivados de algas marinas poseen efectos citotóxicos en células cancerosas humanas (Kang y Kim, 2013; Fan y col., 2014). Por ejemplo, el undecapéptido VECYGPNRPQF, aislado de los residuos de proteína de *Chlorella vulgaris,* presentó una fuerte actividad antiproliferativa en células de cáncer gástrico AGS sin afectar a los fibroblastos de pulmón WI-38 (Sheih y col., 2010). El tetra-péptido CPAP procedente de un hidrolizado de proteínas de *Chlorella pyrenoidosa*

presentó actividad inhibidora de la viabilidad de células de cáncer de hígado humano (HepG2) mediada por lainducción de la apoptosis (Wang y Zhang, 2013). Además, la micro (coacervación compleja) y nanoencapsulación (gelificación ionotrópica) permitió que este péptido resistiera la degradación por las enzimas gastrointestinales.

Péptidos resistentes a la la digestión gastrointestinal *in vitro* liberados a partir de hidrolizados de proteína de soja, han demostrado efecto antitumoral dosis-dependiente en células de cáncer colorrectal (Caco-2 y HCT-116), hepático (HepG2) y pulmonar (NCL-H1299) (Rayaprolu y col., 2013). Sin embargo, estos autores no identificaron las secuencias peptídicas potencialmente responsables de los efectos observados. Uno de los agentes más estudiados por sus propiedades quimiopreventivas es el péptido lunasina procedente de la soja. Este péptido actúa a través de diferentes mecanismos como el bloqueo del ciclo celular, la inducción de la apoptosis e inhibición de la metástasis, demostrado tener efectos antiproliferativos en cultivos celulares de cáncer de próstata, pulmón, mama, linfoma, colorrectal y leucemia (Hernández-Ledesma y Hsieh, 2017). El péptido lunasina también presentó efectos *in vivo* en el modelo de metástasis hepática provocado por células de cáncer colorrectal KM12L4. En estos estudios se observó una reducción del antígeno de proliferación celular, relacionado con el bloqueo del progreso del ciclo celular, y una inducción de la apoptosis mediante modulación de los marcadores Bcl-2:Bax. Sin embargo, se encontraron diferencias entre la administración por vía oral e intraperitoneal del péptido, haciendo difícil obtener resultados definitivos sobre el potencial efecto *in vivo* de la lunasina frente a la metástasis del cáncer colorrectal (Dia y de Mejía, 2011b, 2013). La acción inhibidora de proteasas mostrada por los inhibidores presentes en la soja, como el inhibidor de proteasas Bowman-Birk (BBI) y el inhibidor de tripsina Kunitz (KTI), se ha relacionado con su capacidad anti-cancerígena (Clemente y Arqués, 2014; Cruz-Huerta y col., 2015).

1.3.4 Actividad anti-hipertensiva

Los principales efectos descritos de los péptidos bioactivos sobre el sistema cardiovascular son los relativos a su actividad antitrombótica y antihipertensiva. La

hipertensión arterial es la principal causa de enfermedad en los países industrializados y un grave problema de salud pública (Scirica y col., 2013). Debido a la alta incidencia de esta enfermedad, en la actualidad existen muchos medicamentos con efectos anti-hipertensivos, pero cuyos efectos secundarios han impulsado la búsqueda de terapias eficaces y seguras (Husserl y Messerli, 1981). Entre estas terapias, aquellas basadas en péptidos procedentes de los alimentos se consideran prometedoras por su eficacia como anti-hipertensiva y el pequeño número de efectos secundarios sobre los pacientes (Scirica y col., 2013).

El mecanismo anti-hipertensivo más estudiado para los péptidos bioactivos está mediado por se debe a la inhibición de la actividad de la enzima convertidora de la angiotensina (ECA). El sistema renina-angiotensina-aldosterona desempeña un papel fundamental en el mantenimiento del tono vascular frente a la resistencia periférica. La renina, producida a partir del aparato yuxtaglomerular de los riñones, degrada el angiotensinógeno para producir el decapéptido inactivo angiotensina I. Este último se degrada, por acción de la ECA, liberándose el potente vasoconstrictor angiotensina II. La angiotensina II también estimula la síntesis y liberación de aldosterona de la corteza suprarrenal, lo cual aumenta la presión sanguínea promoviendo la retención de sodio (y, por tanto, la retención de agua) en los túbulos distales (Ahnfelt- Ronne, 1991). La ECA actúa simultanéamente en el sistema quinina-calicreína catalizando la degradación del nanopéptido bradiquinina (Soffer, 1976), lo cual teóricamente contribuye a los efectos hipertensivos de la actividad de la ECA (Ahnfelt-Ronne, 1991) (**Figura 4).**

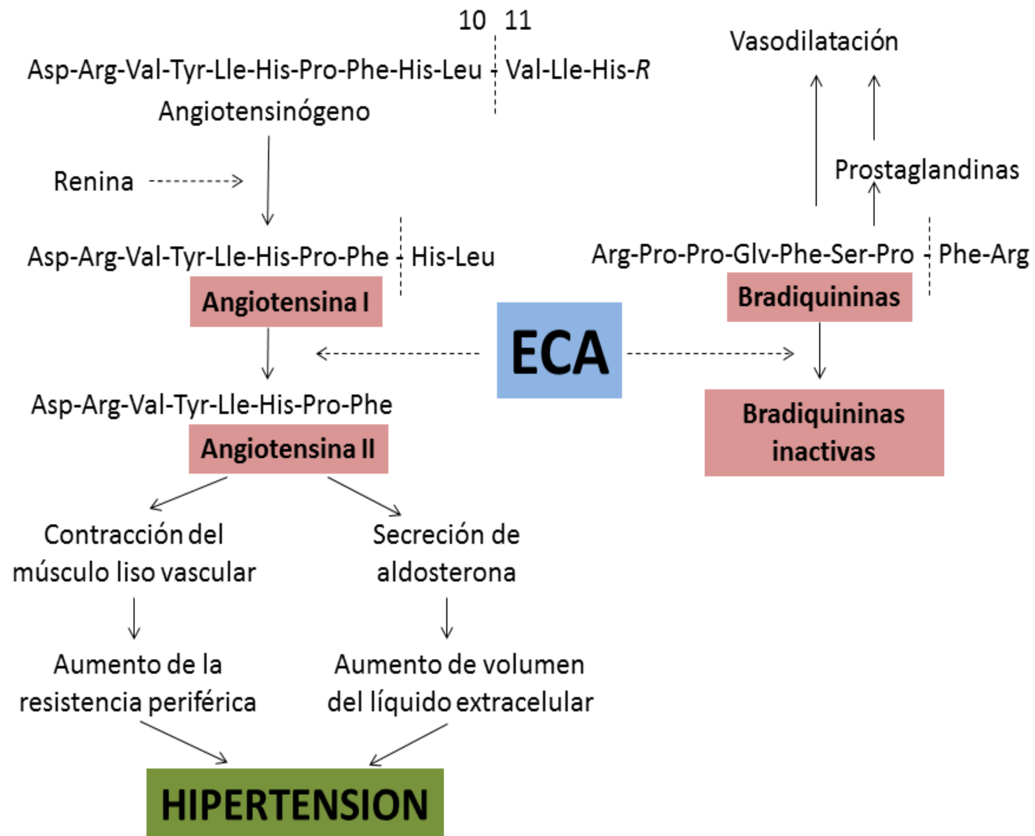


Figura 4. Mecanismo de acción de la enzima convertidora de angiotensina (Tomada de Hansen y col., 1995 y Hansen y col., 1996a).

Se ha descrito actividad inhibidora de la ECA en péptidos derivados de diferentes vegetales. El péptido IAP fue aislado de un hidrolizado de gliadina de trigo preparado con proteasa ácida (Motoi y Kodama, 2003), mientras que un hidrolizado de germen de trigo obtenido con una proteasa alcalina produjo el tripéptido inhibidor de la ECA, cuya secuencia era IVY (Matsui y col., 2002). Nogata y col. (2009) identificaron seis péptidos (LQP, IQP, LRP, VY, IY y TF) inhibidores de la ECA, producidos por la acción de proteasas aspárticas en subproductos de la molienda del trigo. Li y col. (2007) hidrolizaron las proteínas de arroz con la enzima Alcalasa®, obteniendo un péptido, cuya secuencia fue TQVY, y que mostró capacidad para disminuir la presión arterial en ratas espontáneamente hipertensas (REH) después de una administración oral única. Muchos péptidos antihipertensivos se han obtenido por hidrólisis enzimática

de proteínas de colza. Los péptidos IY, VW, RIY, VWIS (Marczak y col., 2003) y LY,TF, RALP (He y col., 2013a) se liberaron tratando las proteínas de colza con Alcalasa®, mientras que el tri-péptido GHS se obtuvo tras la hidrólisis secuencial de dichas proteínas con pepsina y pancreatina (He y col., 2013b). Un hidrolizado del gluten de maíz con pescalasa (una serina proteasa *de Bacillus licheniformis*), fue la fuente del péptido PSGQYY, con un aminoácido hidrofóbico en el amino terminal, un residuo de aminoácido básico en el centro y una tirosina en el carboxilo terminal. A una dosis de 30 mg/Kg de peso corporal, se observó un afecto antagonista a la respuesta supresora de la angiotensina I en ratas, disminuyendo la presión arterial (Suh y col., 1999). Yang y col. (2007) aislaron el di-péptido AY a partir del gluten de maíz hidrolizado con Alcalasa®. El péptido no fue afectado por la preincubación con la ECA, resistiendo la digestión gastrointestinal tras su administración por vía oral a REH. Se observó una reducción de la presión arterial sistólica de 9,5 mm Hg después de 2 horas de su administración oral a dosis de 50 mg/Kg de peso corporal (Yang y col., 2007). Cuatro péptidos (MRWRD, MRW, LRIPVA y IAYKPAG) fueron aislados de la proteina rubisco presente en la espinaca. El efecto antihipertensivo dosis-dependiente de estos péptidos se confirmó en REH (Yang y col., 2003). Péptidos obtenidos de las proteínas hidrolizadas de sésamo con termolisina, mostraron actividad inhibitoria de la ECA, disminuyendo significativamente la presión sistólica en REH con una sola administración (1 y 10 mg/Kg). Siete péptidos inhibidores de la ECA fueron identificados y aislados a partir de este sustrato: LSA, LQP, LKY, IVY, VIY, LVY y MLPAY (Nakano y col., 2006). Dos péptidos activos de las proteínas de guisante amarillo, WMP y ADMFPF fueron obtenidos usando termolisina (Aluko y col., 2014). Posteriormente, se identificaron tres péptidos más, de secuencias LTFPG, IFENLQN y FEGTVFENG con actividad inhibidora de la ECA y la renina (Aluko y col., 2015). Estos péptidos fueron administrados oralmente a REH a dosis de 30 mg/Kg de peso corporal. El péptido LTFPG mostró la disminución más rápida de la presión sistólica con un máximo de -37 mm Hg después de 2 horas de la ingesta. Por el contrario, los efectos máximos de IFENLQN (-37 mm Hg) y FEGTVFENG (-25 mm Hg) fueron observados después de 4 horas de administración (Aluko y col., 2015).

Recientemente se han sintetizado dos péptidos (LRY y VYRT) previamente obtenidos por hidrólisis con una combinación de las enzimas, termolisina, pepsina y quimotripsina de las proteínas de *Palmaria palmata* (alga roja). El tri-péptido LRY tuvo una actividad *in vitro* notablemente alta, seguida por el tetra-péptido VYRT. La actividad de LRY fue equivalente a la del péptido LVY obtenido del sésamo, el mismo que se usa en Japón como agente antihipertensivo en bebidas y alimentos. Por lo tanto, sugirieron que el tri-péptido LRY podría ser utilizado como ingrediente para alimentos funcionales (Furuta y col., 2016). Sato y col. (2002) a partir de hidrolizados de wakame con proteasa S “Amano” procedente de *Bacillus stearothermophi*lus aislaron siete péptidos, VY, IY, AW, FY, VW, IW, y IW, que mostraron resistencia a las proteasas gastrointestinales *in vitro*. Entre ellos, los péptidos VY, IY, FY e IW presentaron en REH efectos anti-hipertensivos tras una única dosis por vía oral de 1 mg/kg de peso corporal.

1.3.5 Hidrolizados y péptidos multifuncionales

Los hidrolizados y péptidos de origen alimentario que presentan dos o más actividades biológicas simultáneamente se conocen como multifuncionales. Memarpoor-Yazdi y col., (2012) han evaluado la actividad antioxidante de la lisozima de clara de huevo de gallina hidrolizada con papaína, tripsina y una combinación de las dos enzimas, mediante la eliminación de radicales DPPH y ABTS, la quelación de iones metálicos y la inhibición de la peroxidación lipídica. El hidrolizado obtenido mediante la combinación de las dos enzimas presentó la mayor actividad antioxidante en comparación con los otros hidrolizados y se eligió para el aislamiento de péptidos. Identificaron el péptido NTDGSTDYGILQINSR que además de activida antioxidante, mostró efectos antimicrobianos tanto en bacterias Gram-negativas como Gram-positivas. Los valores mínimos de concentración del péptido para la inhibición de las bacterias fueron de 355 y 442 µg/mL, respectivamente. Con respecto a las proteínas de origen vegetal, Vernaza y col., (2015) valoraron el efecto de la germinación en combinación con la hidrólisis con Alcalasa® de la variedad de soja brasileña BRS 133. Todos los hidrolizados de las proteínas mostraron una inhibición significativa sobre marcadores inflamatorios tales como el óxido nítrico, óxido nítrico sintasa inducible

(22,8-93,6%), prostaglandina E_2 (64,0-88,3%), ciclooxigenasa-2 (36,2-76,7%) y el factor de necrosis tumoral-α (93,9-99,5%) en macrófagos RAW 264.7 inducidos por LPS. Además, una combinación de setenta y dos horas de germinación e hidrólisis con Alcalasa® durante una hora, dio como resultado la formación de compuestos bioactivos con una actividad antioxidante determinada por el método ORAC más potente y una mejora en la reducción de algunos de los marcadores de inflamación. Por otro lado, se evaluó la actividad antioxidante y anti-hipertensiva de los hidrolizados de proteínas de judía amarga usando Alcalasa®. Los péptidos liberados presentarón capacidad de captación de radicales DPPH (2,9 mg GAE/g), poder redutor FRAP (11,7 mM) y actividad inhibidora de la ECA (80,2%). Se identificaron un total de 29 secuencias peptídicas a partir de la fracción < 10 kDa como los posibles contribuyentes a las actividades observadas (Siow y Gan (2013). El estudió de los hidrolizados de las proteínas de semilla de judía alada con papaína, dio como resultado la producción de biopéptidos con propiedades antioxidantes e inhibidoras de la ECA. La inhibición de la ECA alcanzó su actividad más alta (78,5%) después de doce horas de proteólisis, mientras que la actividad antioxidante, determinada mediante la eliminación de radicales DPPH y la quelación de iones metálicos, alcanzaron valores de 65,0 y 65,7% a las ocho horas y catorce horas, respectivamente. Finalmente, identificaron dos secuencias peptídicas (YPNQKV y FDIRA) con actividad inhibitoria de la ECA y actividad antioxidante (Yea y col., 2014).

Taniguchi y col. (2017) identificaron cinco péptidos catiónicos a partir de las proteínas del salvado de arroz hidrolizadas con pepsina. Tres péptidos fueron químicamente sintetizados (LRRHASEGGHGPHW, EKLLGKQDKGVIIRA y SSFSKGVQRAAF). Entre ellos, el péptido LRRHASEGGHGPHW exhibió actividad antimicrobiana contra *Candida albicans* con un valor de IC_{50} de 289 μM, mientras que los péptidos EKLLGKQDKGVIIRA y SSFSKGVQRAAF presentaron actividad antimicrobiana contra *Porphyromonas gingivalis*, con valores de IC_{50} de 75,6 μM) y 78,5 μM, respectivamente. Además, evaluaron la capacidad para neutralizar LPS. Los tres péptidos inhibieron la actividad endotóxica de LPS de una manera concentración dependiente. Los valores de IC_{50} fueron de 1,07, 0,86 y 1,41 μM para

LRRHASEGGHGPHW, EKLLGKQDKGVIIRA y SSFSKGVQRAAF, respectivamente. El estudio de Siow y col. (2016) identificó y evaluó los péptidos derivados de las proteínas de semilla de comino hidrolizadas con Protamex. Para el ensayo de eliminación de radicales DPPH, los valores de IC_{50} de los péptidos FFRSKLLSDGAAAAKGALLPQYW, RCMAFLLSDGAAAAQQLLPQYW y DPAQPNYPWTAVLVFRH fueron de 0,04, 0,002 y 0,05 µM, respectivamente, así como de 0,02, 0,04 y 0,03 para la actividad inhibidora de la α-amilasa.

En la **Tabla 2** se recogen ejemplos de hidrolizados y péptidos derivados de proteínas de origen vegetal con actividad multifuncional.

Tabla 2. Ejemplos de hidrolizados y péptidos procedentes de proteínas vegetales con actividad multifuncional

Especie vegetal	Enzimas	Actividad biológica	Péptido responsable	Referencias
Soja	Pepsina y pancreatina	Antioxidante Inhibición de la ECA	VAWWN LQSGDALRVPSGTTYY LNSGDALRVPSGTTYY	Puchalska y col., 2014
Soja	Alcalasa®	Antioxidante Anti-inflamatoria	---	Vernaza y col., 2012
Aislado comercial de proteína de guisante (Propulse ™)	Termolisina	Inhibición de la ECA Inhibición de la renina	LTFPG IIPLEN IFENLQN FEGTVFENG	Aluko y col., 2015b
Garbanzo	Pepsina y pancreatina	Antioxidante Inhibición de la ECA	ALEPDHR TETWNPNHPEL FVPH SAEHGSLH	Torres-Fuentes y col., 2011
Frijol alado	Papaína	Antioxidante Inhibición de la ECA	YPNQKV FDIRA	Yea y col., 2014
Salvado de arroz	Pepsina	Antimicrobiana Neutralizante de LPS Angiogénica Hemolítica	LRRHASEGGHGPHW EKLLGKQDKGVIIRA SSFSKGVQRAAF	Taniguchi y col., 2017
Judía caupi	Flavourzima	Antioxidante Inhibición de la ECA	---	Segura-Campos y col., 2010

Judía Negra Jamapa	Alcalasa y Flavourzyme Pepsina y Pancreatina	Antioxidante Inhibición de la ECA	---	Betancur-Ancona y col., 2014
Judía Azufrado Higuera	Alcalasa Termolisina Pancreatina	Antioxidante Inhibición de la ECA	---	Valdez-Ortiz y col., 2012
Judía común	Pepsina y pancreatina	Antioxidante Inhibición de la ECA	GLTSK LSGNK GEGSGA MPACGSS MTEEY	Luna-Vital y col., 2015a
Judía Africana yam	Alcalasa®	Antioxidante Inhibición de la ECA Inhibición de la renina	---	Ajibola y col., 2013)
Judía Amarga	Alcalasa®	Antioxidante Inhibición de la ECA	---	Siow y Gan, 2013
Judías de las variedades Pinto Saltillo, Azufrado Higuera y Plus Black	Alcalasa y Flavourzyme	Antioxidante Antimicrobiana Inhibición de la ECA	---	Ariza-Ortega y col., 2014
Colza	---	Opioide Inhibición de la ECA	RIY (Rapakinin)	Yamada y col., 2011
Semilla de comino	Promatex	Antioxidante Inhibición de la enzima α-amilasa	FFRSKLLSDGAAAAKGALLPQYW RCMAFLLSDGAAAAQQLLPQYW DPAQPNYPWTAVLVFRH	Siow y col., 2016

ECA: enzima convertidora de angiotensina

1.4 Proteínas de quinua y amaranto como fuente de péptidos biactivos

Un número limitado de estudios han demostrado el papel de las proteínas de la quinua como fuente de péptidos bioactivos. Aluko y Monu (2003) describieron la capacidad antioxidante e inhibitoria de la ECA de un hidrolizado de proteína de quinua con Alcalasa®. El hidrolizado fue fraccionado mediante ultrafiltración usando membranas de 10 y 5 kDa. Las propiedades funcionales del concentrado proteico, el hidrolizado y las fracciones se compararon a diferentes valores de pH. La fracción con péptidos de bajo peso molecular presentó la mejor actividad de eliminación de radicales DPPH y la mayor capacidad de inhibición de la ECA. Más recientemente, Nongonierma y col. (2015) han demostrado la capacidad antioxidante e inhibitoria de la enzima DPP-IV de los hidrolizados de proteína de quinua con papaína y papaína microbiana. Estos hidrolizados presentaron valores similares de IC_{50} para DPP-IV y ORAC. Sin embargo, las secuencias responsables de los efectos observados en dichos estudios no han sido identificadas.

El número de estudios enfocados a evaluar el potencial de las proteínas de amaranto como fuente de péptidos bioactivos es más elevado respecto a los encontrados en las proteínas de quinua, aunque mayoritariamente han estudiado las especies *A. hypochondriacus* y *A. mantegazzianus.* (**Tabla 3**). En cuanto al *A. hyponcondriacus* se han identificado varios péptidos inhibidores de la ECA derivados de la globulina 11S (Vecchi y Añón, 2009) y la albúmina 1 tras un tratamiento de hidrólisis con Alcalasa® (Tovar-Pérez y col., 2009). Algunos péptidos han demostrado tener una alta actividad inhibidora de la DPP-IV y por tanto un potencial antidiabético, especialmente tras ser liberados por procesos simulados de digestión gastrointestinal (Velarde-Salcedo y col., 2013). Sabbione y col. (2016) han identificado péptidos con actividad antitrombótica liberados durante la simulación de la digestión gastrointestinal. La fracción más activa de este hidrolizado presentó un IC_{50} de 0,07mg/mL. Montoya-Rodríguez y González de Mejía (2015) evaluaron en modelos celulares el efecto inhibidor de los péptidos sobre marcadores que promueven la aterosclerosis. Así, el péptido HGSEPFGPR redujo la expresión de proteínas asociadas con la vía de

señalización de las lipoxigenasas-1. Además, se ha identificado el péptido quimiopreventivo lunasina en proteínas de amaranto, con una mayor concentración en la fracción de glutelina (3,0 µg/g), pero también presente en las fracciones de albúmina, prolamina y globulina (Silva-Sánchez y col., 2008; Maldonado-Cervantes y col., 2010).

Los digeridos gastrointestinales de las proteínas de *A. mantegazzianus* han demostrado la capacidad de actuar frente a diferentes especies reactivas presentes en el cuerpo humano, especialmente frente a radicales peroxilo e hidroxilo y peroxinitritos (Orsini Delgado y col., 2016). Otro estudio indica la actividad antiproliferativa que presentan péptidos procedentes de lectinas y una fracción hidrofóbica del aislado de proteína de *A. mantegazzianus*, capaces de inhibir la proliferación de células derivadas del osteosarcoma de ratas UMR106 (Quiroga y col., 2015). La hidrólisis de las proteínas de amaranto con Alcalasa® dio lugar a la liberación de secuencias peptídicas con propiedades anti-inflamatorias, como el péptido SSEDIKE, que modula los mediadores IgE en alergias alimentarias (Moronta y col., 2016). Un estudio *in vivo* demostró que la adición de aislado proteico de amaranto (2,5%) a la dieta en un modelo de ratas mejoró el estatus antioxidante y redujo el nivel de colesterol en plasma e hígado tras 28 días, con un aumento de la excreción fecal del colesterol como posible mecanismo de acción (Lado y col., 2015). Conforti y col. (2005) demostraron que las semillas de dos variedades de *A. caudatus* (Oscar blanco y Victor Red) poseían actividad antidiabética vía inhibición de la α-amilasa. Hasta el momento, no hemos encontrado estudios que evalúen la multifuncionalidad de péptidos derivados de *A. caudatus* tras procesos de digestión gastrointestinal simulada.

Tabla 3. Péptidos bioactivos identificados a partir de proteínas de amaranto

Especie	Enzimas	Proteína	Actividad biológica	Péptido responsable	Referencias
Amaranthus cruentus	Pepsina y pancreatina	---	Hipocolesterolémico	LVG GGV IVG VGVI/VGVL	Soares y col., 2015
Amaranthus mantegazzianus	Pepsina y pancreatina	11S Globulina	Antioxidante	AWEEREQGSR YLAGKPQQEH IYIEQGNGITGM TEVWDSNEQ	Orsini Delgado y col., 2016
	---	Lectina	Anti-proliferativa en modelo celular UMR106	Polipéptidos	Quiroga y col., 2015
	Digestión *in vivo*	---	Antioxidante Anti-colesterol	---	Lado y col., 2015
Amaranthus hypochondriacus	Tripsina	Glutelina	Inhibidora de la enzima DPP-IV	AP PPLP GKP LF FP VY	Silva-Sánchez y col., 2008; Maldonado-Cervantes y col., 2010
	Pepsina y pancreatina	11S Globulina	Inhibidora de la enzima DPP-IV	IPI IPA PPPP GP	Velarde-Salcedo y col., 2013

Amaranthus hypochondriacus	Alcalasa®	Albúmina AmA1	Inhibición de la ECA	---	Tovar-Pérez y col., 2009
	Alcalasa®	11S Globulina	Inhibición de la ECA	ALEP VIKP IKP LEP	Vecchi y Añón, 2009; Tovar-Pérez y col., 2009
	Alcalasa®	Concentrado proteico	Inmunomoduladora Anti-inflamatoria en modelo celular Caco-2	SSEDIKE IADEDPDEANDK DNDEE KPV LENAIDKK	Moronta y col., 2016

ECA: enzima convertidora de angiotensina
DPP-IV: dipeptidil peptidasa IV

1.5 Efecto de la digestión en la liberación y biodisponibilidad de péptidos con actividad biológica

La digestión gastrointestinal es el conjunto de reacciones por las cuales se produce la degradación de los alimentos y su transformación en moléculas de tamaño reducido, siendo un proceso esencial para la salud de los seres vivos. Las macromoléculas se degradan a moléculas más pequeñas que pueden ser luego absorbidas por el organismo. La digestión supone primero una rotura mecánica y luego una degradación enzimática, tanto de glúcidos como de proteínas y lípidos, por medio de enzimas de la saliva, jugo gástrico, jugo pancreático y células de las vellosidades intestinales (Guerra y col., 2012). De manera general, en la cavidad oral se produce en primer lugar la masticación de los alimentos, que se mezclan con la saliva, donde la enzima amilasa cataliza la hidrólisis del almidón en azúcares. Las proteínas no se hidrolizan en esta primera etapa, siendo transportadas en forma de bolo alimenticio a través del esófago al estómago, donde entra en contacto con proteasas, principalmente pepsina y lipasas. La digestión de las proteínas comienza en el estómago **(Figura 5)**, bajo la acción de la enzima pepsina y en condiciones de acidez, dando lugar a la liberación de polipéptidos y algunos aminoácidos. El quimo es gradualmente desplazado al intestino delgado, donde el pH del estómago es neutralizado y se incorporan jugos digestivos pancreáticos (con enzimas digestivas como la tripsina, elastasa y quimiotripsina junto con exopeptidasas como la carboxipeptidasa A y B) y de la vesícula biliar (ácidos biliares), generando péptidos de dos a seis residuos aminoacídicos así como aminoácidos libres. La etapa final en la digestión de las proteínas alimentarias ocurre en la superficie de los enterocitos por la acción de las proteasas de las células intestinales de borde en cepillo (Shimizu, 2004; Arhewoh 2005).

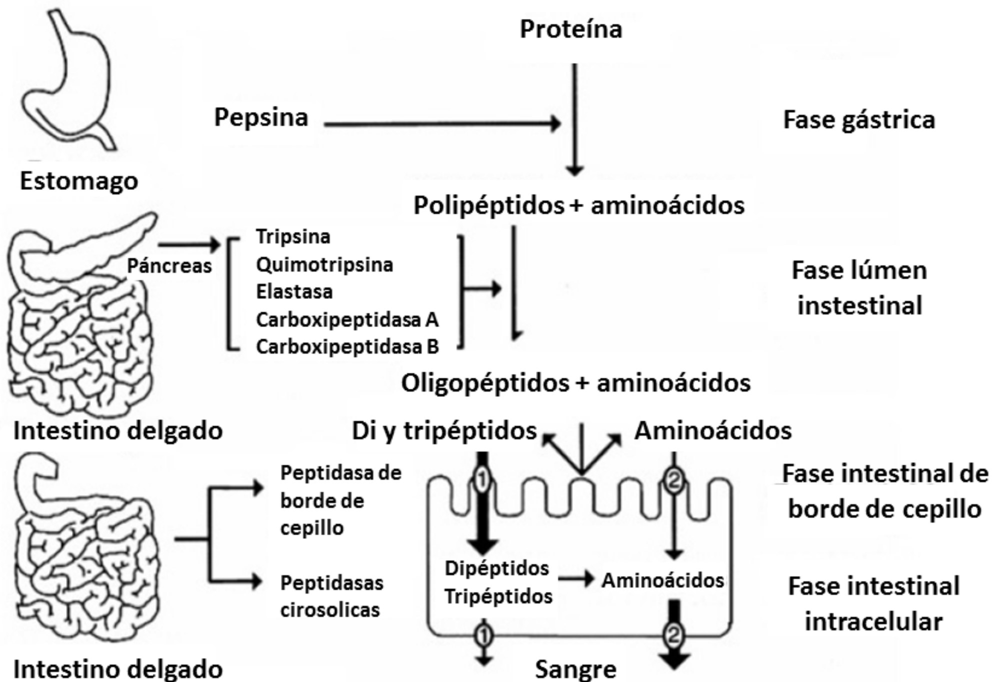


Figura 5. Activación o inactivación de las proteínas durante la digestión y absorción gastrointestinal (Tomada de Segura-Campos y col., 2010)

El estudio del proceso digestivo de las proteínas es esencial para conocer los distintos péptidos que se forman y su interacción con el organismo. Mediante el análisis de la digestión *in vivo* de los alimentos, ya sea en humanos o animales, se obtienen los resultados más relevantes. Sin embargo, suponen un mayor tiempo y coste, además de las implicaciones éticas que conlleva. Por ello, la digestión gastrointestinal *in vitro* simulando condiciones fisiológicas, es una herramienta muy útil para evaluar la liberación y estabilidad de los péptidos frente a las enzimas digestivas (Matsui y col., 2002). La simulación del tracto gastrointestinal supone establecer unos parámetros, lo más cercanos posibles a las condiciones fisiológicas de pH y tiempos de tránsito para cada sistema, así como las condiciones químicas y enzimáticas para cada tiempo. Una regla básica en la simulación gastrointestinal es conseguir la relación adecuada de complejidad técnica frente a la relevancia fisiológica, ya que siempre existirán parámetros que quedarán fuera del modelo, como el sistema inmune, mecanismos

hormonales y la microbiota. Los modelos de digestión gastrointestinal *in vitro* se clasifican en estáticos y dinámicos, siendo estos últimos los más complejos ya que simulan cambios, tanto físicos, como vaciado gástrico, absorción de nutrientes y movimientos peristálticos, como cambios químicos, modificación de pH y empleo de secreciones a lo largo del tiempo de digestión. Esto hace que los modelos estáticos sean los más empleados. Sin embargo, debido a la variación entre los resultados obtenidos mediante los diferentes métodos utilizados surgió en el ámbito de la Acción Europea COST Action "*Improving heath properties of food by sharing our knowledge on the digestive process*" (Infogest) la necesidad de consensuar un protocolo de digestión estática *in vitro* (Minekus y col., 2014). En el se emulan las fases oral, gástrica e intestinal de la digestión, utilizando en cada etapa fluidos simulados con la composición química y enzimática adecuada y el pH y tiempo necesarios.

Los péptidos liberados durante el proceso de digestión o que han resistido al mismo y llegan intactos al intestino, bien pueden ejercer una función local como relajantes de la mucosa gástrica o inhibidores de la secreción de enzimas, o bien pueden atravesar el epitelio intestinal y pasar al torrente sanguíneo para alcanzar los órganos diana y actuar a nivel sistémico (Arhewoh, 2005). Sin embargo, las diferencias metabólicas y las variaciones anatómicas, fisiológicas y bioquímicas que se presentan en las diferentes partes del sistema digestivo repercuten en la absorción de los mismos (Lee, 2002). Se han hallado péptidos liberados durante la digestión gastrointestinal *in vitro* de semillas de soja y leche de soja. Los resultados indicaron que las proteínas de soja experimentaron una degradación durante la digestión, generando un gran número de péptidos bioactivos, algunos con actividad antimicrobiana (Singh y col., 2014). La hidrólisis de la proteína de girasol con pepsina y pancreatina permitió la liberación del péptido inhibidor de la ECA, FVNPQAGS (Megías y col., 2004). Tres di-péptidos, IR, KF y EF, obtenidos de la digestión gastrointestinal *in vitro* del aislado de proteína de guisante, demostraron una fuerte inhibición de la ECA y la renina, con mayor potencia frente a la ECA que frente a la renina (Li y Aluko, 2010). En otro estudio, se liberaron los péptidos IHRF y RF a partir de la glutelina del arroz digerida con quimotripsina. En particular, el péptido IHRF presentó actividad vasodilatadora en la arteria mesentérica

de REH. Al ser administrado por vía oral, el tetra-péptido IHRF disminuyó la presión arterial sistólica en REH y su actividad anti-hipertensiva fue más potente y de larga duración que la del di-péptido RF (Kagebayashi y col., 2012; Kontani y col., 2014). Dos tri-péptidos (VNP y VWP) se obtuvieron por hidrólisis de proteínas de arroz con Alcalasa® y tripsina (Chen y col., 2013). Estos péptidos fueron inhibidores competitivos de la ECA, y estables frente a las proteasas gastrointestinales, pepsina y quimotripsina. Además, la administración oral única de estos tripéptidos en REH disminuyó significativamente la presión sistólica con efectos anti-hipertensivos durante 8 horas (Chen y col., 2013). La digestión de la soja con pepsina reveló actividad inhibitoria de los péptidos IA, YLAGNQ, FFL, IYLL y VMDKPQG. Su actividad antihipertensiva fue confirmada en REH que fueron alimentadas por soluciones de la fracción peptídica en polvo (0,9% p/v; 2,0 g/Kg de peso corporal). Se observó una reducción significativa de la presión arterial (17,5 mmHg) después de 2 horas, y su efecto continuó durante 6 horas después de la administración oral (Chen y col., 2002). Cuatro tetra-péptidos activos (AIYK, YKYY, KFYG, y YNKL) fueron aislados de las proteínas de la alga wakame (*Undaria pinnatifida*) por procesos de digestión *in vitro.* Cada péptido se sintetizó y su efecto antihipertensivo se confirmó después de la administración oral (50 mg/Kg) en REH (Suetsuma y Nakano, 2000). El efecto antihipertensivo del wakame se evaluó en pacientes hipertensos donde se observó una disminución significativa de la presión sistólica, después de la administración oral diaria de 3,3 g de wakame durante 4 semanas (Nakano y col., 1998) Recientemente, Dave y col. (2016) demostraron incluso que la digestión de proteínas endógenas gastrointestinales también puede producir péptidos bioactivos.

La biodisponibilidad de los péptidos depende de la actividad enzimática de las diferentes áreas del tracto gastrointestinal. La primera barrera de los péptidos es el lumen del intestino delgado, el cual contiene gran cantidad de proteasas secretadas por el páncreas y proteasas de las células de la mucosa. La segunda barrera enzimática son las proteasas de la membrana del borde en cepillo de las células epiteliales, las cuales contienen al menos quince peptidasas que en conjunto tienen una amplia especificidad pudiendo degradar tanto proteínas como péptidos. Tras su administración

oral, una proteína o péptido bioactivo debe resistir la acción de al menos 40 diferentes enzimas durante su paso por el intestino delgado. Además, las peptidasas lisosomales cuyo número se estima en más de 60, también representa una barrera para los péptidos y proteínas que son transportados por las células epiteliales vía endocitosis (Segura-Campos y col., 2010). En este contexto, y con la finalidad de comprender los mecanismos de absorción intestinal de los biopéptidos que permitan establecer estrategias para maximizar su asimilación y potencial activo, se viene empleando la línea de células (Caco-2), procedentes de carcinoma de colon rectal humano que presentan actividades enzimáticas y mecanismos de transporte similares a los del epitelio del intestino delgado (Elimrani y col., 2003). De esta manera Satake y col., (2002) demostraron que la ruta paracelular era el principal mecanismo de transporte del tri-péptido antihipertensivo VPP, aunque una pequeña cantidad del mismo se absorbe transcelularmente vía el transportador PepT1. Hsieh y col. (2010) demostraron la biodisponibilidad del péptido lunasina en ratones y ratas, evidenciando su presencia en varios tejidos como riñon, hígado, glándula mamaria, pulmón, y próstata. De la misma manera, se ha determinado la presencia de este péptido de forma intacta y bioactiva en el plasma e hígado de ratas alimentadas con dietas de trigo (Jeong y col., 2007a), cebada (Jeong y col., 2010), centeno (Jeong y col., 2009) y soja (Jeong y col., 2007c) enriquecidas con lunasina.

2. RESULTS/RESULTADOS

Caracterización de las proteínas de quinua y evaluación de su potencial como fuente de péptidos multifuncionales tras su digestión gastrointestinal.

2.1 Publicación I: **Analysis of protein isolation from quinoa (*Chenopodium quinoa* Willd.).**

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH, 9 (2016) 332-334

2.2 Publicación II: **Release of dipeptidyl peptidase IV, α-amilase and α-glucosidase inhibitory peptides from quinoa (*Chenopodium quinoa* Willd.) during *in vitro* simulated gastrointestinal digestion.**

JOURNAL OF FUNCTIONAL FOODS, 35 (2017) 531-539

2.3 Publicación III: **Chemopreventive properties of peptides released from quinoa (*Chenopodium quinoa* Willd.) protein under simulated gastrointestinal digestion.**

FOOD RESEARCH INTERNATIONAL (*submitted*)

2.4 Publicación IV: **Digestibility of quinoa *(Chenopodium quinoa* Willd.) protein concentrate and its potential to inhibit lipid peroxidation in the zebrafish larvae model.**

PLANT FOODS FOR HUMAN NUTRITION, 72 (2017) 294-300

Publicación I. Análisis del aislado proteico de quinua (*Chenopodium quinoa* Willd).

A. Toapanta, C. Carpio, R. Vilcacundo, W. Carrillo. 2016. Analysis of protein isolate from quinoa (*Chenopodium quinoa* Willd). Asian Journal of Pharmaceutical and Clinical Research, 9, 332-334.

Resumen

El uso de aislados proteicos en la industria alimentaria se ha incrementado en los últimos años debido a ciertos factores como son el alto contenido en proteína y su buena funcionalidad, la presencia de compuestos bioactivos y el bajo contenido en factores anti-nutricionales. Actualmente, el método más usado para obtener estos aislados se basa en una extracción a pH alcalino tras la solubilización de las proteínas a pH ácido logrando así la separación isoeléctrica de las mismas. Por lo tanto, el objetivo de este estudio fue obtener aislados de proteína de quinua empleando un pH alcalino y diferentes pHs de precipitación. Los aislados fueron analizados mediante electroforesis. El rendimiento del proceso de obtención del aislado fue del 6,29% a pH 4,0 de precipitación de las proteínas. El contenido proteico fue superior al 64% en todos los aislados obtenidos. Tras el análisis electroforético, pudieron observarse las bandas correspondientes a albúminas y globulinas, fracciones proteicas mayoritarias en este pseudocereal. La banda con peso molecular de 60 kD correspondió a la globulina 7S, mientras que las bandas de peso molecular 33-36 kDa y 20-22 kDa correspondieron a la globulina 11S y la banda de peso molecular inferior a 15,4 kDa correspondió a las albúminas.

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Research Article

ANALYSIS OF PROTEIN ISOLATE FROM QUINOA (*CHENOPODIUM QUINOA* WILLD)

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ABSTRACT

Objective: The aim of this study was to obtain protein isolate from quinoa using alkaline pH at different pHs of precipitation and to analyze protein isolate with electrophoresis.

Methods: Quinoa protein isolates were obtained using isoelectric precipitation method at different pHs. Proteins were analyzed using electrophoresis native-polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate - PAGE.

Results: A yield of 6.29% of protein isolate of defatted quinoa at pH 4.0 was obtained. The content of protein isolate was higher than 64% in all pH assays. Globulins and albumins in protein isolate at different pHs were observed. One band near 130 kDa was found. A band with MW 60 kDa corresponding to 7S globulin was found. The bands, MW 33-36 kDa and MW 20-22 kDa, correspond to 11S globulin. Bands less to 15.4 kDa correspond to albumins.

Conclusions: Quinoa is a good source of proteins. Globulins and albumins were identified in the quinoa protein isolate.

Keywords: Quinoa, Globulins, Albumins, Polypeptides, Protein isolate.

INTRODUCTION

Quinoa (*Chenopodium quinoa* Willd) is a pseudo-cereal native of the Andean regions of South America and belongs to the family Chenopodiaceae [1,2]. It was the main crop of the Incas, a cereal-like crop with high yield seed. Quinoa has been selected by FAO, 2014 as one of the crops destined to offer food security in the 21st century, quinoa plants are tolerant to salinity and drought stress, and able to grow in marginal regions [3]. The seed protein content is high (about 12-15%), and its essential amino acid balance is excellent due to a wider amino acid spectrum than cereals and legumes, with higher lysine (5.1-6.4%) and methionine (0.4-1.0%) contents. The use of protein isolate has increased in the food industry because of different factors such as higher protein level, good functionality, bioactive components, and lower content of anti-nutritional factors [4]. The most used method to obtain protein isolate is alkaline pH (8-11) through solubilization of proteins at acid pH (4-6) for their isoelectric precipitation [5]. The aim of this study was to obtain protein isolate from quinoa using alkaline pH at different pHs of precipitation and to analyze these proteins with electrophoresis sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE).

METHODS

Quinoa flour and proximate analysis

Quinoa flour was defatted through extraction with hexane (1:10 w/v) at room temperature during 24 hrs, under continuous stirring during the first 5 hrs. After drying at room temperature, the flour was stored at 4°C until used. Analytical methods such as moisture, fat, total fiber, and soluble solids contents were determined according to the methods of AOAC (2012) [6], numbers 9250.10, 930.09, 985.29, and 923.03, respectively. The protein content of the sample was determined by the micro-Kjeldahl method AOAC number 920.152, % (N × 6.25). Carbohydrates percentage was calculated with the formulas: % carbohydrates = 100 - (% moisture + % proteins + % fat +% soluble solids + % total fiber). Contents were expressed on a dry weight basis.

Protein isolate from quinoa

Quinoa isolate was prepared according to Martinez and Añón (1996) [7] with modifications. The defatted flour was suspended in water in a 1:10 w/v, and the suspension was adjusted to pH 8.0 by adding 2 M NaOH. The suspension was stirred during one hour and then centrifuged at 4,500 g for 30 minutes at 25°C. The supernatant was adjusted to pHs 2.0; 3.0; 4.0; 5.0; and 6.0 with 2 N HCl and centrifuged for 20 minutes at 4,500 g. The pellet was suspended in a small volume of water, neutralized with 0.1 M NaOH, and lyophilized and then frozen at −20°C. The content of protein isolate was determined using the method Biuret [8].

SDS-PAGE

Native-PAGE and SDS-PAGE electrophoresis of quinoa protein isolate were carried out according to the method proposed by Laemmli (1970) [9] using 4-8% and 4-12% polyacrylamide gel in a Mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, USA). Polypeptide bands were stained in Coomassie Brilliant Blue G-250 for 12 hours. Relative molecular masses of protein were determined by a comparison to molecular weight (MW) markers (Bio-Rad, Hercules, CA, USA) and software quantity one of chemidoc (Bio-Rad).

RESULTS

Composition analysis

Table 1 shows the approximate composition of defatted quinoa flour obtained with water. The protein content was 13%; this result is in accordance with other authors [4,10,11]. Table 2 shows the protein yields from quinoa protein isolate. At pH 4.0, the protein yield obtained was the highest with 6.29% of protein isolate. The content of protein increased from 13% in the quinoa flour to 84.32% in the proteins isolate at pH 2.0. In all pHs, the content of proteins was higher than 60% (Table 2).

Effect of pH on the extraction of quinoa proteins

Quinoa seed shows proteins fractions of globulins and albumins as storage protein. Albumins and globulins are the highest protein fractions (44-77% of total, respectively) while the percentage of prolamins is low (0.5-7.0%) [12]. Globulins have two groups depending on its sedimentation coefficient: 11-12 S and 7-8 S. Quinoa storage protein predominant are globulins 11S and 7S. Recently, globulin 11S from quinoa has been named Chenopodin. This protein has two

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Table 1: Proximate analysis of DQF

%	Protein	Fat	Moisture	Total fiber	Soluble solids	Carbohydrates
DQF	13.0±0.1	4.99±0.01	9.05±0.03	1.01±0.1	2.09±0.03	69.9±0.4

DQF: Defatted quinoa flour, SD: Standard deviation, results represent the average of three determinations±SD

Table 2: Content of quinoa protein isolate obtained at different pHs

Sample	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0
% isolate	3.37±0.10	3.82±0.04	6.29±0.01	5.66±0.01	3.93±0.07
% protein	84.32±2.5	82.76±0.07	65.01±0.04	73.65±1.09	64.78±0.55

Values are expressed in grams per 100 g of protein. Values are means±SD of three determinations, SD: Standard deviation

subunits consisting of an acid polypeptide (AS) (32-39 kDa) and a basic polypeptide (AB) (22-23 kDa). The 2S albumin has been described as a band of low MW near 6-8 kDa [5,13,14].

Electrophoresis pattern

Quinoa isolate proteins were compared using electrophoresis Native-PAGE, and six similar protein profile (Fig. 1) were found by native-PAGE in all pHs assays with high expression at pH 4, 5, and 6.

SDS-PAGE

Electrophoresis SDS-PAGE at reduced and non-reduced conditions of quinoa-extracted protein in water at different pHs are shown in Fig. 2. Proteins mass was determined with software Quantity one of Chemidoc™ PM (Bio-Rad). In the presence of 2-β-Mercaptoethanol, proteins with high MW MW130 kDa were not found in all pHs, whereas proteins with 60 kDa corresponding to 7S globulin according to Abugoch *et al.* (2008) [5] were found in all pHs. Proteins with MW 33-36 kDa correspond to 11S AS were found in all pHs with high expression. On the other hand, proteins with MW 20-22 kDa corresponding to 11S AB were found in all pH values but with higher expression in pHs 4, 5, and 6. Proteins with 20-36 kDa correspond to Chenopodin according to Abugoch *et al.* (2008) [5]. All proteins bands <14.4 kDa corresponding to albumin components according to Brinegar *et al*. (1996) [14] were found in high expressions in pHs 5 and 6.

SDS-PAGE without 2-β-Mercaptoethanol present similar profile of proteins at all pHs assays, the band with MW 50 kDa was found in all pHs with high expression. Proteins between 28 kDa and 36 kDa have high expression in all pHs (Fig. 3).

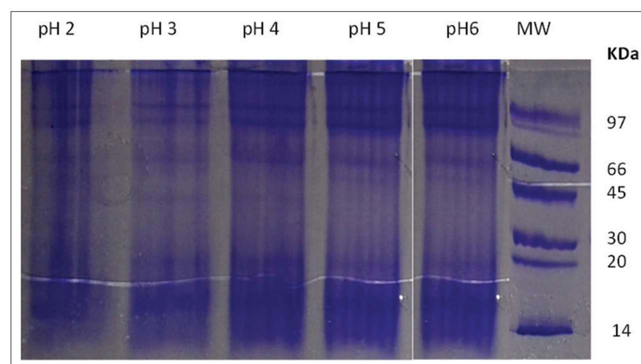

Fig. 1: Electrophoresis native-polyacrylamide gel electrophoresis profiles of quinoa proteins obtained at different pHs molecular weight marker

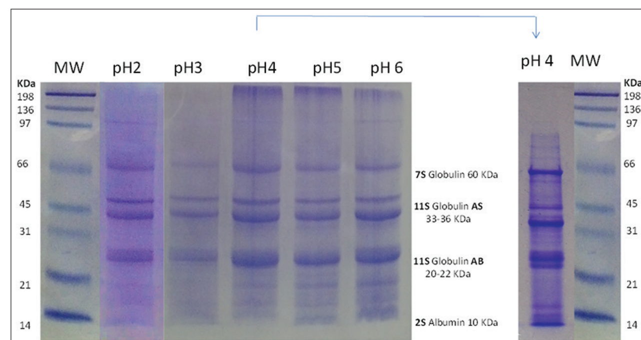

Fig. 2: Electrophoresis sodium dodecyl sulfate - polyacrylamide gel electrophoresis SDS-PAGE profiles of quinoa proteins obtained at different pHs of precipitation extracted under reducing condition (SDS+ 2-β-Mercaptoethanol) molecular weight marker

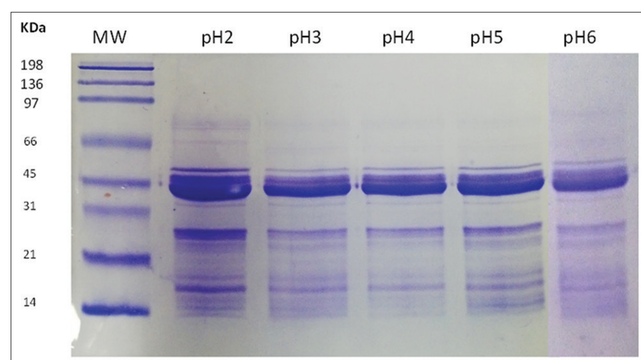

Fig. 3: Electrophoresis sodium dodecyl sulfate - polyacrylamide gel electrophoresis profiles of quinoa proteins obtained at different pHs of precipitation extracted without reducing conditions, molecular weight marker

DISCUSSION

Srivastava *et al*., 2013 [15] indicated the composition and degree of unfolding of protein isolates are regulated by specific or selecting different combinations of extraction and precipitation pH. We observed this comportment in the quinoa proteins. It knows that two of the major type of storage proteins in legume and some no legume seeds are 7S and 11S based on its sedimentation coefficients. Quinoa seeds, due to its high protein content, are actually the subject of many investigations as a potential food source and functional food [16].

CONCLUSION

The content of proteins was higher than 64% in all pHs assays. Albumins and globulins were identified in quinoa protein isolates using isoelectric precipitation at different pHs. Quinoa is a good candidate for supplementation of food protein or substitution of common cereal grains and can be a source of bioactive components.

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Publicación II: Liberación de péptidos inhibidores de la dipeptidil peptidase IV, α-amilasa y α-glucosidasa a partir de proteína de quinua (*Chenopodium quinoa* Willd.) during un proceso de digestión *in vitro* simulando las condiciones gastrointestinales.

R. Vilcacundo, C. Martínez-Villaluenga, B. Hernández-Ledesma. 2017. Release of dipeptidyl peptidase IV, α-amilase and α-glucosidase inhibitory peptides from quinoa (*Chenopodium quinoa* Willd.) during *in vitro* simulated gastrointestinal digestion. Journal of Functional Foods, 35, 531-539

Resumen

La diabetes es considerada como una de las principales causas de mortalidad y morbilidad. Por ello, la investigación enfocada en la búsqueda y desarrollo de nuevos alimentos con actividad anti-diabética se ha intensificado en los últimos años. En este estudio, se ha evaluado la influencia de la digestión gastrointestinal de proteína de quinua en la liberación de péptidos con potencial anti-diabético. Para ello, la proteína de quinua presente en un concentrado proteico fue sometida a un proceso de digestión *in vitro* simulando condiciones fisiológicas humanas. Tras la digestión, los digeridos obtenidos durante la fase gástrica y la fase duodenal fueron fraccionados mediante ultrafiltración y evaluados en cuanto a su actividad inhibidora de las enzimas dipeptidil peptidasa IV, α-amilasa y α-glucosidasa. Los péptidos liberados durante la fase duodenal fueron los que mostraron los efectos más potentes. Se han identificado tres nuevos péptidos derivados de la proteína de almacenamiento globulina 11S en la fracción más activa obtenida mediante HPLC a escala semipreparativa. Estos péptidos mostraron la capacidad para inhibir las enzimas implicadas en la degradación de las hormonas incretinas y en la digestión de los carbohidratos. Por lo tanto, tras estos resultados, la proteína de quinua podría considerarse como un prometedor ingrediente de nuevos alimentos funcionales destinados al control de la diabetes.

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Release of dipeptidyl peptidase IV, α-amylase and α-glucosidase inhibitory peptides from quinoa (*Chenopodium quinoa* Willd.) during *in vitro* simulated gastrointestinal digestion

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ABSTRACT

As diabetes is a major cause of mortality and morbidity in epidemic rates, continuous research is being done on development of foods with anti-diabetic activity. In this study, the influence of gastrointestinal digestion of quinoa protein to release peptides with anti-diabetic potential was investigated. Quinoa protein was subjected to an *in vitro* simulated gastrointestinal digestion and fractionated by ultrafiltration. Gastric and gastroduodenal digests and peptide fractions were evaluated for dipeptidyl peptidase IV (DPP-IV), α-amylase and α-glucosidase inhibitory activities. Peptides released during the duodenal phase showed the highest inhibitory effects. Three novel peptides derived from 11S seed storage globulin B were identified in the most active fraction by HPLC-MS/MS. These peptides showed ability to inhibit enzymes involved in incretin degradation and digestion of dietary carbohydrates. Therefore, quinoa proteins are promising ingredients of functional foods or nutraceutical applications for the control of diabetes.

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1. Introduction

Type-2 diabetes mellitus (T2DM) is a chronic metabolic disorder considered as one of the major global health problems because of its rising incidence and prevalence rates (WHO, 2016). T2DM consists of impaired insulin secretion and activity, and eventual pancreatic β-cells failure. Insulin resistance leads to an increase in blood glucose levels with the outcome of postprandial hyperglycemia (De Fronzo, 1999; Robertson, 1995). T2DM affects carbohydrate, protein and fat metabolism, and it is the leading cause of cardiovascular and neurological disorders, blindness, kidney failure, and lower limb amputation (Jao et al., 2015). Various strategies are being used for the prevention and management of T2DM such as changes in the diet avoiding processed foods and increasing the intake of fruits and vegetables, regular physical exercise, and the use of different antidiabetic drugs (Patil, Mandal, Kumar Tomar, & Anand, 2015). However, because of the adverse side effects and limited long-term durability associated with insulin and conventional oral anti-hyperglycaemic agents, natural and safe approaches need to be explored to prevent further progression of T2DM and its related disorders (Majumdar & Inzucchi, 2013). One of the most recent approaches is through inhibition of dipeptidyl peptidase IV (DPP-IV). This enzyme is responsible for degradation and inactivation of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Both gut incretin hormones are secreted in response to the presence of nutrients in the intestinal lumen, and act by stimulating glucose-dependent insulin secretion in the pancreatic β-cells (Holst & Deacon, 2004; Mentlein, 2005). Continuous intravenous infusion of GLP-1 regularizes blood glucose levels in diabetic individuals (Nauck et al., 1993). However, because of the rapid degradation and inactivation of GLP-1 in plasma by DPP-IV, the effects of this incretin hormone are short-lasting (Nauck et al., 1996). Animal models and human trials have demonstrated that specific DPP-IV inhibition increases the half-life of total circulating GLP-1, reduces plasma glucose, and enhanced glucose tolerance (Deacon, Hughes, & Holst, 1998; Deacon, Nauck, Meier, Hücking, & Holst, 2000; Mitani, Takimoto, Hughes, & Kimura, 2002). Thus, specific enzyme inhibitors have emerged over the past few years as a new class of oral agents for the treatment of T2DM (Hunziker, Henning, & Peters, 2005; McIntosh, Demuth, Pospisilik, & Pederson, 2005). Furthermore, blocking carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase is another alternative in the control

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of glucose homeostasis in diabetic patients (Johnson, Lucius, Meyer, & de Mejia, 2011). Salivary and pancreatic α-amylase inhibitors are known to block hydrolysis of complex starches to oligosaccharides, and to prevent starch glucose moieties from being absorbed by the human body (Richardson, 1991). Furthermore, α-glucosidase inhibitors block hydrolysis of di-, tri- and oligosaccharides to glucose in the small intestine. Thereby, inhibition of both enzymes decreases the rate of digestion of carbohydrates and, as a result, less glucose is absorbed and transported into the circulation (El Abed et al., 2017).

Over the last two decades, food-derived bioactive peptides have attracted much attention for their potential to serve as natural alternatives or complements to synthetic drugs. Bioactive peptides, embedded within the sequence of the precursor protein, can be released by gastrointestinal digestion and/or processing technologies. Once released, they have been demonstrated to exert a plethora of biological activities improving human health and reducing risk of chronic disorders. Due to their chemical structure, peptides can interact with amino acids in the catalytic site of enzymes involved in important diseases, inhibiting their action (Mojica & de Mejia, 2016). As it has been previously reviewed, multiple short peptides derived from animal and plant sources have been recognized as DPP-IV inhibitors (Jao et al., 2015). Studies on some of these peptides have demonstrated that they are effective stimulating insulin secretion and improving glycemic control in animal models and subjects with T2DM (Mochida, Hira, & Hara, 2010; Power, Hallihan, & Jakeman, 2009; Uenishi, Kabuki, Seto, Serizawa, & Nakajima, 2012). In the case of α-amilase and α-glucosidase inhibitors of peptidic nature, the studies are more limited, although recently, the inhibitory potential of peptides derived from black bean (Mojica & de Mejia, 2016) or hard-to cook bean proteins (Oseguera-Toledo, Gonzalez de Mejia, & Amaya-Llano, 2015) has been demonstrated.

Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal traditionally consumed by Andean cultures that, in the last years, has been introduced in high-income countries of Europe and North America, attracting the interest of the consumers. This fact has been linked to its gluten-free nature, as well as to its content of protein, fiber, vitamins and minerals, fatty acids, and multiple phytochemicals that provide quinoa a remarkable advantage over other grains in terms of human nutrition and health (Vilcacundo & Hernández-Ledesma, 2017). In addition to their higher content than other dietary grains, quinoa proteins have been accepted as high quality protein because of their balanced essential amino acids pattern. Some beneficial effects such as cholesterol-lowering activity have also been attributed to quinoa protein fractions in animal models (Takao et al., 2005). However, only a limited number of studies have demonstrated the role of quinoa proteins as source of bioactive peptides. Aluko and Monu (2003) described the antioxidant and angiotensin-converting enzyme inhibitory properties of an Alcalase® hydrolyzate of quinoa protein. More recently, Nongonierma, Le Maux, Dubrulle, Barre, and FitzGerald (2015) reported the DPP-IV and antioxidant activity of quinoa protein hydrolyzates with papain. Though, the sequences responsible for the observed effects have not been identified yet. Moreover, no studies have focused on the potential of gastrointestinal digestion to release bioactive peptides from quinoa proteins. Therefore, the aim of this study was to evaluate the DPP-IV, α-amylase and α-glucosidase inhibitory activity of quinoa protein concentrate *in vitro* hydrolyzed under conditions simulating gastrointestinal digestion. Different peptide fractions were isolated from the most active gastroduodenal digest, their main peptides identified, and the subsequent synthetic peptide sequences evaluated for their contribution on the demonstrated anti-diabetic properties.

2. Materials and methods

2.1. Materials

Pepsin from porcine gastric mucosa (3200–4500 U/mg protein, EC 3.4.23.1), pancreatin from porcine pancreas (4 × USP, EC 232-468-9), porcine bile extract, Pefabloc® SC, the tripeptide IPI (I9759), called diprotin A, human recombinant DPP-IV enzyme (D3446), porcine pancreatic α-amylase (EC 3.2.1.1) and rat intestine α-glucosidase (EC 3.2.1.20) were purchased from Sigma-Aldrich (Madrid, Spain). Chromogenic substrate (H-Gly-Pro-p-nitroaniline; P188) was purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA).

2.2. Obtention of quinoa protein concentrate

The quinoa protein concentrate (QP) was prepared following the protocol of Toapanta, Carpio, Vilcacundo, and Carrillo (2016) with slight modifications. *Chenopodium quinoa* Willd. (quinoa) flour (Mascorona, Ambato, Ecuador), once defatted, was suspended in water (1:10, w/v), adjusting the pH of suspension to 8.0 with 2 M NaOH. After stirring the suspension during 1 h, it was centrifuged at 4500*g* for 30 min at 25 °C. The supernatant was adjusted to pH 4.0 with 2 N HCl, and centrifuged at 4500*g* during 20 min at 4 °C. The pellet was dissolved in a small volume of water, neutralized with 0.1 M NaOH, lyophilized, and kept at −20 °C until further analysis. The protein content of quinoa PC was determined by Kjeldahl.

2.3. In vitro simulation of gastrointestinal digestion of quinoa protein

The quinoa PC was digested according to the *in vitro* harmonized protocol (Minekus et al., 2014). Briefly, 520 mg of PC were dissolved in 5 mL H_2O and then, the mixture was diluted at a ratio of 50:50 (v/v) with simulated gastric fluid (pH 3.0) containing pepsin from porcine gastric mucosa (2000 U/mL of digest). Samples were withdrawn at the starting point and after 120 min of gastric digestion, stopping the reaction by adjusting the pH at 7.0 with NaOH 1 M and snap freezing in liquid nitrogen. Gastric phase was mixed with the same volume of simulated intestinal fluid (pH 7.0) containing pancreatin from porcine pancreas (100 U trypsin activity/mL of final mixture) and porcine bile extract (10 mM in the final mixture). All simulated fluids were tempered at 37 °C before use. Digestions were performed in duplicate by incubating at 37 °C in an orbital shaker at 150 rpm. Samples of gastroduodenal digests were withdrawn after 60 and 120 min incubation, and the digestion was stopped with Pefabloc® SC (5 mM) and snap freezing. Samples were freeze-dried and kept at −20 °C until analysis. A digestion blank consisting of a mixture of enzymes used in digestions at the same concentration without quinoa PC was prepared. Actual enzyme activities and bile concentration were measured according to the assays described in the protocol (Minekus et al., 2014).

Digests were subjected to ultrafiltration through a hydrophilic 5000 Da cutoff membrane (Agilent Technologies, Inc., Waldbronn, Germany). Fractions with molecular weight higher and lower than 5 kDa were freeze dried and kept at −20 °C until use. The protein content of digests and peptidic fractions was determined by the bicinchoninic acid method (BCA) (Pierce, Rockford, IL, USA) using bovine serum albumin as standard protein.

2.4. SDS-PAGE

Samples were dissolved at a concentration of 1 mg of protein/mL in sample buffer that contained Tris-HCl (0.05 M, pH 6.8,

Sigma-Aldrich), SDS (1.6%, w:v, Merck, Darmstadt, Germany), glycerol (8%, v:v, Panreac Química SAU, Castellar del Vallés, Barcelona, Spain), β-mercaptoethanol (2%, v:v, Sigma-Aldrich) and bromophenol blue indicator (0.002%, w:v, Merck), heated at 95 °C for 5 min and loaded on 12% Bis-Trispolyacrilamide gels (Criterion_XT, Bio-Rad, Hercules, CA, USA). Electrophoretic separations were run at 100 V for 5 min and then at 150 V, using the XT MES running buffer (Bio-Rad) in the criterion cell (Bio-Rad). A molecular weight marker (Precision Plus Protein™ Unstainend standard, Bio-Rad) containing ten *Strep*-tagged recombinant proteins (10–250 kDa), including three reference bands (25, 50, 75 kDa was used. Gels were stained with Coomasie Blue (Instant blue, Expedeon, Swavesey, UK) and images were taken with a Molecular Imager® Versa-Doc™ MP 5000 system (Bio-Rad) and processed with Quantity One® 1-D analysis software (Bio-Rad).

2.5. Assessment of the anti-diabetic activity

2.5.1. Measurement of the DPP-IV inhibitory activity

DPP-IV inhibitory activity was measured in 96-well plates following the protocol described by Silveira, Martínez-Maqueda, Recio, and Hernández-Ledesma (2013). Recombinant soluble human DPP-IV (0.26 mU per test well; 15 μL) was incubated at 37 °C in the absence or presence of different concentrations of samples (final volume 50 μL per well) for 10 min. Diprotin A was used as positive control. A volume of 50 μL of the assay buffer containing H-Gly-Pro-p-nitroaniline was added to each well at final concentration of 100 μM. Absorbance was read at 405 nm in a microplate reader (BMG Labtech Inc., Champigny sur Marne, France) at 2 min time intervals starting from 0 up to 30 min. Recorded data were plotted versus time. The best fit straight line was obtained in the time range over which the increase in the absorbance was linear. Then, data were expressed as % remaining activity in the presence of test samples versus control (without sample) and represented versus protein/peptide concentration. Each sample was analyzed three times. Data were plotted and fitted to logarithmic regression to obtain dose-response curves. The results were expressed as IC_{50} value or protein/peptide concentration needed to inhibit 50% of DPP-IV activity.

2.5.2. α-amylase inhibitory activity

The α-amylase inhibition assay was adapted from a previously described method (Johnson et al., 2011). Briefly, 50 μL of sample, positive control (2 mM acarbose) or negative control (distilled water) were added to 100 μL α-amylase solution (2 U/mL in 0.02 M sodium phosphate buffer pH 6.9). Test tubes were incubated at 20 °C in a Thermomixer™ orbital shaker (Eppendorf Iberica, Madrid, Spain) for 5 min. Later, 100 μL of 1% soluble starch solution (previously dissolved in 0.02 M sodium phosphate buffer pH 6.9 and boiled for 15 min) were added to each tube and incubated in a Thermomixer™ orbital shaker (Eppendorf Iberica) at 20 °C, 1000 rpm for 6 min. Finally, 100 μL of dinitrosalicylic acid color reagent were added, and the tubes were placed in a 100 °C water bath for 15 min. A volume of 800 μL of distilled water were added to the mixture, and the absorbance was read at 540 nm in a Synergy HT microplate reader (Biotek, Winooski, VT, USA). Percent inhibition was calculated relative to the negative control having 100% enzyme activity.

2.5.3. α -glucosidase inhibitory activity

α-glucosidase inhibition assay was performed following a previous method (Satoh, Igarashi, Yamada, Takahashi, & Watanabe, 2015) with slight modifications. Briefly, 100 μL of sample, positive control (1 mM acarbose) or negative control (distilled water) were added to 50 μL of rat intestine α-glucosidase (1 U/mL in 0.1 M maleate buffer pH 6.9). Test tubes were incubated at 37 °C for 5 min. After pre-incubation, 50 μL of substrate (2 mM maltose or 20 mM sucrose) were added to each tube. The reaction mixtures were incubated using a Thermomixer™ orbital shaker (Eppendorf Iberica) at 37 °C, 1000 rpm for 30 min. Finally, reactions were stopped placing the tubes in a water bath at 100 °C for 5 min. Supernatants were collected by centrifugation at 20 °C, 12,000*g* for 5 min and stored at −20 °C until glucose quantification. Glucose concentration of reaction mixtures was measured using the Amplex® Red glucose/glucose oxidase assay kit (Invitrogen, Madrid, Spain). Absorbance was measured using a Synergy HT plate reader (Biotek) at 560 nm. Glucose concentration was calculated using a linear standard curve (0–200 μM) from a freshly prepared 400 mM stock solution. Percent inhibition was calculated relative to the negative control having 100% enzyme activity.

2.6. Separation of peptides by semi-preparative RP-HPLC

Semi-preparative RP-HPLC was carried out according to Hernández-Ledesma, Miralles, Amigo, Ramos, and Recio (2005) with some modifications. Separation was performed on a HPLC system (Waters, Mildford, MA, USA) equipped with two pumps (module Delta 600), a pump controller (module 600), an autosampler (module 717) and a diode array detector (module 996). The data-processing software was Millenium version 32 (Waters). Digest was dissolved in distilled water at a concentration of 10 mg/mL, and the injection volume was 400 μL. A Hi-Pore Reversed Phase RP-318 (250 × 21.5 mm) column (Bio-Rad) was used. Fractions were eluted at a flow rate of 10 mL/min, with a linear gradient of solvent B (acetonitrile: trifluoroacetic acid (TFA), 1000:0.8, v/v) in A (water:TFA, 1000:1, v/v) going from 0% to 60% B in 45 min, 60% to 100% B in 5 min, 10 min with 100% B and from 100% B to 0% B in 30 min. Each chromatographic run was repeated 45–50 times and the fractions were collected automatically with a Fraction Collector (Model II, Waters). The collected fractions were pooled, frozen and lyophilized. The peptide content of collected fractions was determined by the BCA method.

2.7. Identification of peptides by RP-HPLC-MS/MS

RP-HPLC coupled to tandem mass spectrometry (RP-HPLC-MS/MS) analysis of collected fractions was performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik GmbH, Bremen, Germany) and equipped with an electrospray ionization source. The column used was a reverse phase Mediterranea Sea C_{18} Column (150 × 2.1 mm i.d., 5 μm particle size) (Teknokroma, Barcelona, Spain). Peptides were eluted with a linear gradient of solvent B (acetonitrile:formic acid, 1000:1, v/v) in A (water:formic acid, 1000:1, v/v) going from 0% in 45% in 60 min at a flow rate of 0.2 mL/min. The injection volume was 50 μL. Using Data Analysis™ (version 3.0; Bruker Daltonics), the *m*/*z* spectral data were processed and transformed to representing mass values. The acquired MS/MS spectra were interpreted using BioTools (version 2.1; Bruker Daltonics).

2.8. Peptide synthesis

Synthetic peptides were provided by Chengdu KaiJie Biopharm Co., Ltd (Chengdu, Sichuan, P. R. China) that synthesized them by the conventional Fmoc solid-phase synthesis method. The purity of synthetic peptides, determined by HPLC-MS analysis, was higher than 96.5%.

2.9. Statistical analysis

Data represent the mean and standard deviation of three independent experiments (n = 3). Data were subjected to one-way analysis of variance (ANOVA) to compare experimental values using Statgraphics 5.0 (Statistical Graphics Corporation, Rockville, Md). Comparison between groups was performed using a Duncan's multiple-range test, and differences were considered significant at $P \leq 0.05$.

3. Results and discussion

3.1. Anti-diabetic activity of quinoa protein concentrate digested under simulated gastrointestinal digestion

The analysis of the quinoa PC revealed that it contained 74.10% protein. This value was higher to that (65.01%) recently determined by Toapanta et al. (2016) for quinoa protein extracted at the same pH used in our extraction process. The PC was subjected to an *in vitro* gastrointestinal digestion simulating physiological conditions. Protein patterns obtained by SDS-PAGE of the PC at the starting point of the digestion (D0), the gastric digest after 120 min incubation with pepsin (GD120), and gastroduodenal digests obtained after 120 min incubation with pepsin and 60 min (ID60) and 120 min (ID120) incubation with pancreatin are shown in Fig. 1. Before digestion, the pattern of quinoa proteins was similar to that previously reported in the literature with four major protein bands ranged from 20 to 60 kDa (Fig. 1, lane 1) (Brinegar & Goundan, 1993). Repo-Carrasco, Espinoza, and Jacobsen (2003) indicated that 37% of total quinoa protein is constituted by chenopodin, a globulin 11S-type protein. The 11S-type protein of seed is characterized by having two heterogeneous sets of polypeptides in the size ranged from 30 to 40 kDa (acidic subunits) and 20–25 kDa (basic subunits) which are joined by disulfide bonds in the native protein. In our PC, both acidic (32–39 kDa) and basic (22–23 kDa) subunits were visible for chenopodin (bands B and C, respectively in Fig. 1, lane 1) as it had been previously described (Brinegar, Sine, & Nwokocha, 1996). Besides, non-digested PC showed two major bands (45–60 kDa) as well as bands of molecular weight lower than 15 kDa. According to Abugoch, Romero, Tapia, Silva, and Rivera (2008), quinoa protein band of 60 kDa might correspond to 7S globulin (band A in Fig. 1, lane 1) while bands at the end of the gel might correspond to 2S albumins (Brinegar et al., 1996). During gastric phase, quinoa proteins were partially hydrolyzed by pepsin, decreasing the bands intensity (lane 2 in Fig. 1) while complete protein degradation was observed after their incubation with pancreatin (lanes 3 and 4). Low molecular weight bands were not detected in ID60 and ID120 digests. This finding could be related to an extensive proteolysis and production of small peptides that leached out from the gel. Bands appearing at the gel (lanes 3 and 4) corresponded to enzymes used in simulated digestive process since they were also visible when digestion blank (without PC) was analyzed (lane 5).

Non-digested PC, gastric and gastroduodenal digests were analyzed by their ability to inhibit DPP-IV, α-amylase and α-glucosidase (Table 1). Undigested PC at a concentration of 6.0 mg of protein/mL did not show inhibitory effects against any of three enzymes. After the gastric phase, the digest GD120 showed inhibitory activity against DPP-IV with an IC_{50} value of 2.52 ± 0.06 mg protein/mL, although no effects against α-amylase and α-glucosidase were shown. Similarly, individual whey proteins

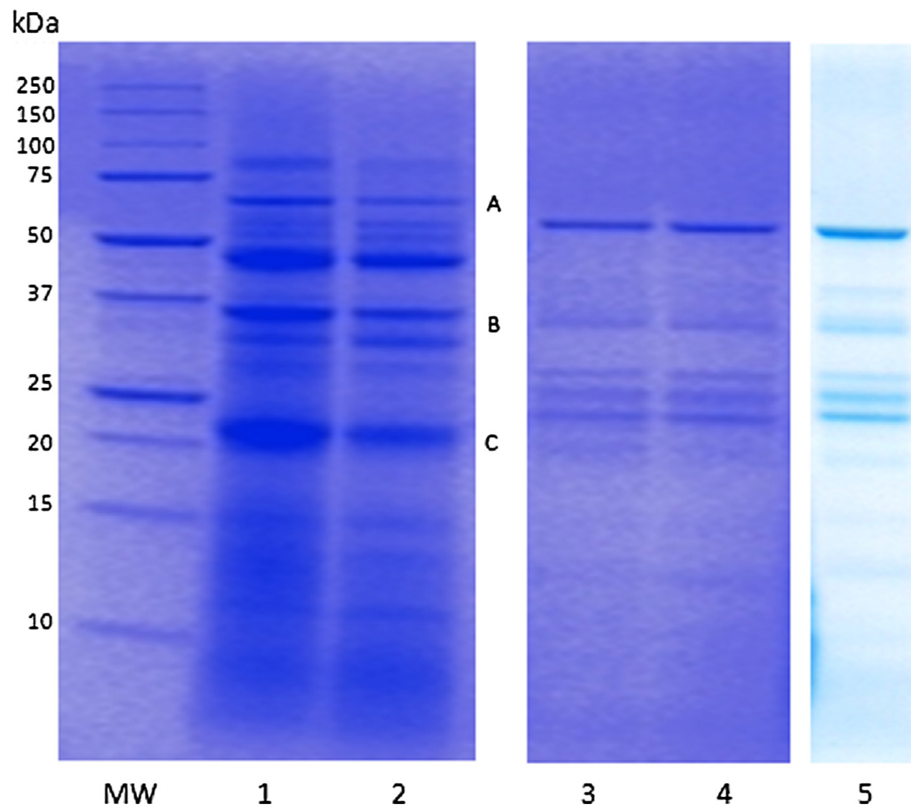

Fig. 1. Characterization of the digests obtained after an *in vitro* simulated gastrointestinal digestion by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. MW: molecular weight marker; Lane 1: digest at time 0 (D0), Lane 2: gastric digest at 120 min (GD120), Lane 3: gastroduodenal digest at 60 min (ID60), Lane 4: gastroduodenal digest at 120 min (ID120), Lane 5: Digestion blank with digestive enzymes. A: 7S globulin (60 kDa), B: 11S globulin acidic subunit (33–36 kDa), C: 11S globulin basic subunit (20–22 kDa).

Table 1
Inhibitory activity of dipeptidyl peptidase-IV (DPP-IV), α-amylase and α-glucosidase (expressed as IC_{50} or protein concentration needed to inhibit 50% original enzyme activity) of quinoa protein concentrate digests obtained after gastric (120 min, GD120) and duodenal (60 and 120 min, ID60 and ID120) phases, and the fractions higher and lower than 5 kDa obtained from digests ID60 and ID120.

Sample	Enzyme inhibitory activity (IC_{50}) (mg protein/mL)		
	DPP-IV[a]	α-amylase[a]	α-glucosidase[a]
D0	n.d.*	n.d.*	n.d.*
GD120	2.52 ± 0.06d	n.d.*	n.d.*
ID60	0.23 ± 0.01a	0.53 ± 0.01b	1.75 ± 0.13b
F > 5 KDa	0.46 ± 0.01b	n.d.+	n.d.+
F < 5 KDa	0.31 ± 0.01a	1.09 ± 0.04c	1.45 ± 0.12a
ID120	0.25 ± 0.01a	0.19 ± 0.02a	1.81 ± 0.03b
F > 5 KDa	0.84 ± 0.07c	n.d.+	n.d.+
F < 5 KDa	0.48 ± 0.03b	0.32 ± 0.01b	1.64 ± 0.05ab

Different lowercase letters in each column indicate significant differences among samples ($P < 0.05$, Duncan test).
n.d.* No inhibitory effects observed at the highest concentration used (6 mg/mL).
n.d.+ No inhibitory effects observed at the highest concentration used (2.5 mg/mL).
[a] Values are the mean ± standard deviation.

digested with pepsin were found to exert potent DPP-IV inhibitory effects with IC_{50} values ranged from 0.04 mg protein/mL (α-lactalbumin digest) to 1.28 mg protein/mL (β-lactoglobulin hydrolyzate) without showing any effect against α-glucosidase (Lacroix and Li-Chan, 2013). Once the gastric digest was subjected to the action of pancreatin, digests ID60 and ID120 showed potent DPP-IV inhibitory activity with similar IC_{50} values of 0.23 ± 0.01 mg protein/mL and 0.25 ± 0.01 mg protein/mL, respectively. These values were lower as compared to IC_{50} reported by Velarde-Salcedo et al. (2013) for amaranth glutelin peptides released during *in vitro* gastrointestinal digestion although higher than those reported for cereal flours derived from highland, oat and buckwheat digested with pepsin and a trypsin-pancreatin mixture (Wang, Yu, Zhang, Zhang, & Fan, 2015). *In vitro* simulated digests obtained from hemp, pea, rice and soy proteins have also been found to inhibit DPP-IV activity with IC_{50} values between 1.85 ± 0.34 and 4.50 ± 0.55 mg dry weight hydrolyzate/mL (Nongonierma & Fitzgerald, 2015). α-amylase inhibitory activity increased during the duodenal phase reaching an IC_{50} value of 0.19 ± 0.02 mg protein/mL at the end of digestive process. To date, few data are available on the α-amylase inhibitory effects of food protein hydrolyzates. Recently, Mojica and de Mejia (2016) have reported 53.4% inhibition for Alcalase® hydrolyzate of black bean proteins at a final concentration of 0.33 mg hydrolyzate/mL. In the case of α-glucosidase inhibitory activity, the first 60 min of incubation with pancreatin were enough to release peptides with inhibitory activity from quinoa proteins, and thus, ID60 showed an IC_{50} value of 1.75 ± 0.13 mg protein/mL that remained constant until the end of digestion (IC_{50} value of 1.81 ± 0.03 mg protein/mL for ID120). This activity was more potent than that determined in alkaline protease hydrolyzate from sardine muscle (IC_{50} value of 48.7 mg/mL, Matsui, Yoshimoto, Osajima, Oki, & Osajima, 1996) or that shown by Alcalase® hemp seed proteins hydrolyzates that reached 50% enzyme inhibition at concentrations higher than 5 mg/mL (Ren et al., 2016). In both assays, authors used microbial α-glucosidases while in our study rat intestinal α-glucosidase was used. Structural variations among microbial and mammalian enzymes could be responsible for the different binding of inhibitors to their active site and thus, for the different levels of inhibitory activity observed. Some synthetic inhibitors have been demonstrated to strongly affect the activity of mammalian α-glucosidase having little inhibitory effects on baker's yeast enzyme (Oki, Matsui, & Osajima 1999). Because of the better relevance to human health, mammalian intestinal α-glucosidase is preferred in studies evaluating the inhibitory activity of food derived peptides. Moreover, maltose is also preferred as substrate for the enzyme assay since it is the major digestive product of starch in the small intestine (Lacroix & Li-Chan, 2013).

In order to determine the influence of the molecular weight of peptides on the anti-diabetic activity of quinoa protein digests, ID60 and ID120 were separated into two molecular weight peptide fractions, >5 kDa and <5 kDa. The inhibitory activity of these fractions against the three enzymes was measured (Table 1). In the case of the DPP-IV inhibitory activity, IC_{50} values of fractions containing short peptides were lower than those of fractions comprising large peptides. This is in agreement with previous studies that have described the DPP-IV inhibitory activity to be associated with lower molecular weight peptides (Power, Nongonierma, Jakeman, & FitzGerald, 2014). However, IC_{50} values of <5 kDa fractions were slightly higher (0.31 ± 0.01 mg protein/mL and 0.48 ± 0.03 mg protein/mL for fractions derived from ID60 and ID120, respectively) than those determined in whole hydrolyzates, indicating that some larger peptides might also contribute to the activity of quinoa protein digests. Velarde-Salcedo et al. (2013) predicted the binding modes of large amaranth glutelin peptides containing 13 residues which are able to interact in the enzyme dimerization site blocking the active dimer formation. Similarly, a fraction containing whey protein-derived peptides in the 3–10 kDa range was found to be the most active inhibiting DPP-IV activity (Babij et al., 2014). Three DPP-IV inhibitory peptides with IC_{50} values between 78 and 116 μM were identified in tuna cooking juice hydrolyzates (Huang, Jao, Ho, & Hsu, 2012). These peptides comprised 13–15 amino acid residues which were much longer than the preferable DPP-IV inhibitory peptides which size ranges from two to five amino acids. These findings made the authors to suggest that the DPP-IV inhibitory activity could be dependent on the composition and sequence of amino acids, in addition to their length. Lacroix and Li-Chan (2012) also indicated that the apparent DPP-IV inhibitory activity in high molecular mass peptides might be attributed to their DPP-IV substrate-like structural features, which could act as substrate analogues to competitively interfere with the combination of the synthetic substrate and DPP-IV.

Fractions <5 kDa, in particular that derived from ID120 showed potent α-amylase inhibitory activity (IC_{50} value of 0.32 ± 0.01 mg protein/mL). Moderate α-glucosidase inhibitory activity was observed for both <5 kDa fractions, with IC_{50} values of 1.45 ± 0.12 mg protein/mL (ID60) and 1.64 ± 0.05 mg protein/mL (ID120). These results indicate that short peptides could be considered as the main responsible for the inhibition of both carbohydrate hydrolyzing enzymes observed for quinoa protein digests released during the duodenal phase. In consistency with our results, previous studies have shown that low molecular weight peptides (<3 kDa) derived from rice bran glutelins (Uraipong & Zhao, 2016) or common bean proteins (Oseguera-Toledo et al., 2015) are more potent inhibiting β-glucosidase.

3.2. Identification of potential anti-diabetic peptides

To identify and characterize peptides potentially responsible for the anti-diabetic activity observed, ID60 was selected and fractionated by semi-preparative RP-HPLC (Fig. 2). In addition to the inhibitory activity shown by this digest towards three enzymes assayed, preliminary studies carried out in our laboratory have demonstrated that ID60 exerts other biological activities (unpublished data) making it an interesting sample to identify bioactive peptides derived from quinoa protein. It was observed that the majority of the peptides eluted between 5 and 45 min. Three fractions, named as F-1, F-2 and F-3, were collected and evaluated for their enzyme inhibitory activity (Table 2). F-1 and F-2 showed inhibitory effects towards the three enzymes while F-3 only showed activity against DPP-IV and α-glucosidase. The most potent effects

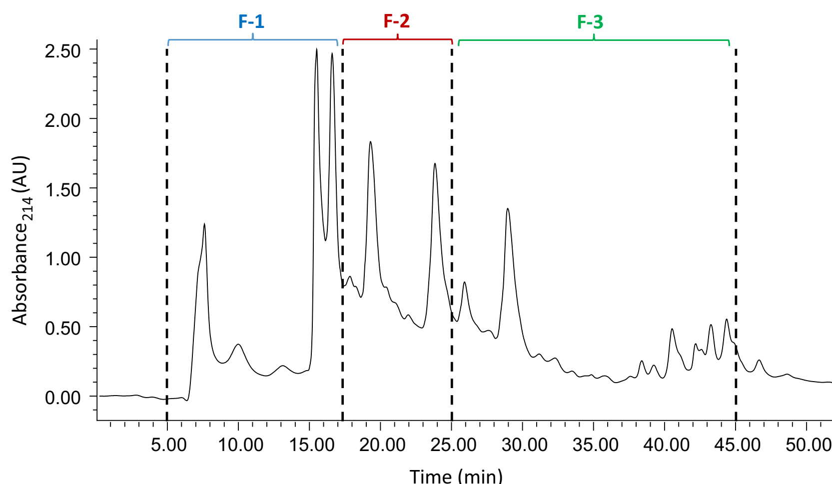

Fig. 2. Fractionation by semi-preparative RP-HPLC of the gastroduodenal digest at 60 min (ID60). Collected fractions are termed with F followed by a number.

Table 2
Inhibitory activity of dipeptidyl peptidase-IV (DPP-IV), α-amylase and α-glucosidase (expressed as IC_{50} or peptide concentration needed to inhibit 50% original enzyme activity) of fractions collected by RP-HPLC from quinoa protein concentrate digested with pepsin for 120 min and pancreatin for 60 min (digest ID60).

HPLC fraction	Enzyme inhibitory activity (IC_{50}) (mg peptide/mL)		
	DPP-IV[a]	α-amylase[a]	α-glucosidase[a]
F-1	0.75 ± 0.04b	0.59 ± 0.02b	2.32 ± 0.08b
F-2	0.41 ± 0.04a	0.42 ± 0.01a	0.86 ± 0.00a
F-3	0.78 ± 0.05b	>3.00	2.87 ± 0.24c

[a] Values are the mean ± standard deviation. Different lowercase (online) letters in each column indicate significant differences among samples (P < 0.05, Duncan test).

were observed for fraction F-2 containing peptides that eluted between 17 and 25 min. As inhibitor of α-amylase and α-glucosidase, the IC_{50} values determined for this fraction: 0.42 ± 0.01 and 0.86 ± 0.01 mg peptide/mL respectively, were lower than those determined in the ID60 digest. However, the IC_{50} value as DPP-IV inhibitor was almost 2-fold the value calculated for the original digest (0.23 ± 0.01 mg peptide/mL), indicating the potential contribution of peptides contained in fractions F-1 and F-3 on the effects of whole ID60 digest.

In order to identify the peptides present in the chromatographic fractions, they were analyzed by MS/MS. With this analysis, many peptide compounds were detected although only seven fragments present in fractions F-2 and F-3 matched quinoa proteins found in the NCBInr database (Table 3). As an example, Fig. 3A shows the mass spectrum of a single-charged ion with *m*/*z* at 871.40, which matched the sequence GEHGSDGNV corresponding to the fragment f (193–201) of 11S seed storage globulin B, by MS/MS sequence interpretation and database searching (Fig. 3B). Other two 11S globulin B-derived peptides were identified in fraction F-2 in which sequences IQAEGGLT (787.41 Da) and DKKYPK (764.40 Da) were identified. Two fragments found in fraction F-3 corresponded to the sequence of maturase K, one corresponded to betaine aldehyde dehydrogenase, and one fragment belonged to starch synthase, chloroplastic/amyloplastic (Table 3).

3.3. Anti-diabetic properties of synthetic peptides

Since fraction F-2 had shown the highest inhibitory activity, the three peptides identified in this fraction were synthesized and assessed for their DPP-IV, α-amylase and α-glucosidase inhibitory activity in order to evaluate their potential contribution on the observed effects. Percentages of enzyme inhibition at a given concentration are shown in Table 4. No inhibitory effects were shown for peptide DKDYPK against DPP-IV activity at the highest concentration (1000 μM) used in the assay. At 80 and 250 μM, peptide GEHGSDGNV did not show inhibitory effects although it reached 20% inhibition at 1061 μM. None of these peptides contain proline at the first, second, third, or fourth N-terminal position that has been defined as a structural feature determinant on the activity of DPP-IV inhibitory peptides (Pieter, 2006). The percentages of inhibition for peptide IQAEGGLT were 7.40 ± 0.50 and 17.05 ± 0.06% at 80 and 250 μM, respectively. The IC_{20} or peptide concentration needed to inhibit 50% of DPP-IV activity was calculated obtaining a value of 267.81 μM. The higher inhibitory effects shown by this peptide could be due to the presence of isoleucine as N-terminal amino acid. Recent *in silico* studies have shown that potent DPP-IV inhibitory peptides generally contain a branched-chain amino acid (leucine, isoleucine, or valine) or an aromatic residue with a polar group in the side-chain (primarily tryptophan) at their N-terminal position (Nongonierma & FitzGerald, 2014; Tulipano, Faggi, Nardone, Cocchi, & Caroli, 2015). A mixture containing three peptides at equimolar concentrations was prepared and evaluated for its DPP-IV inhibitory activity, reaching 30%

Table 3
Peptides identified by HPLC-MS/MS in fractions F-2 and F-3 collected from quinoa protein concentrate digested with pepsin for 120 min and pancreatin for 60 min (digest ID60).

Fraction	Observed mass	Calculated mass	Fragment	Sequence	Source protein	NCBI Accession number
F-2	787.40	787.41	26–33	IQAEGGLT	11S Globulin B	ABI94736.1
	764.40	764.37	163–168	DKDYPK	11S Globulin B	ABI94736.1
	870.40	870.35	193–201	GEHGSDGNV	11S Globulin B	ABI94736.1
F-3	811.30	811.41	23–28	IFQEY	Maturase K	CCI55135.1
	758.40	758.40	224–229	SFFVFL	Maturase K	CCI55135.1
	958.50	958.49	469–476	RELGEWGI	Betaine aldehyde dehydrogenase	ALA65446.1
	953.50	953.52	15–25	GGLGDVLGGLP	Starch synthase, chloroplastic/amyloplastic	ALB36792.1

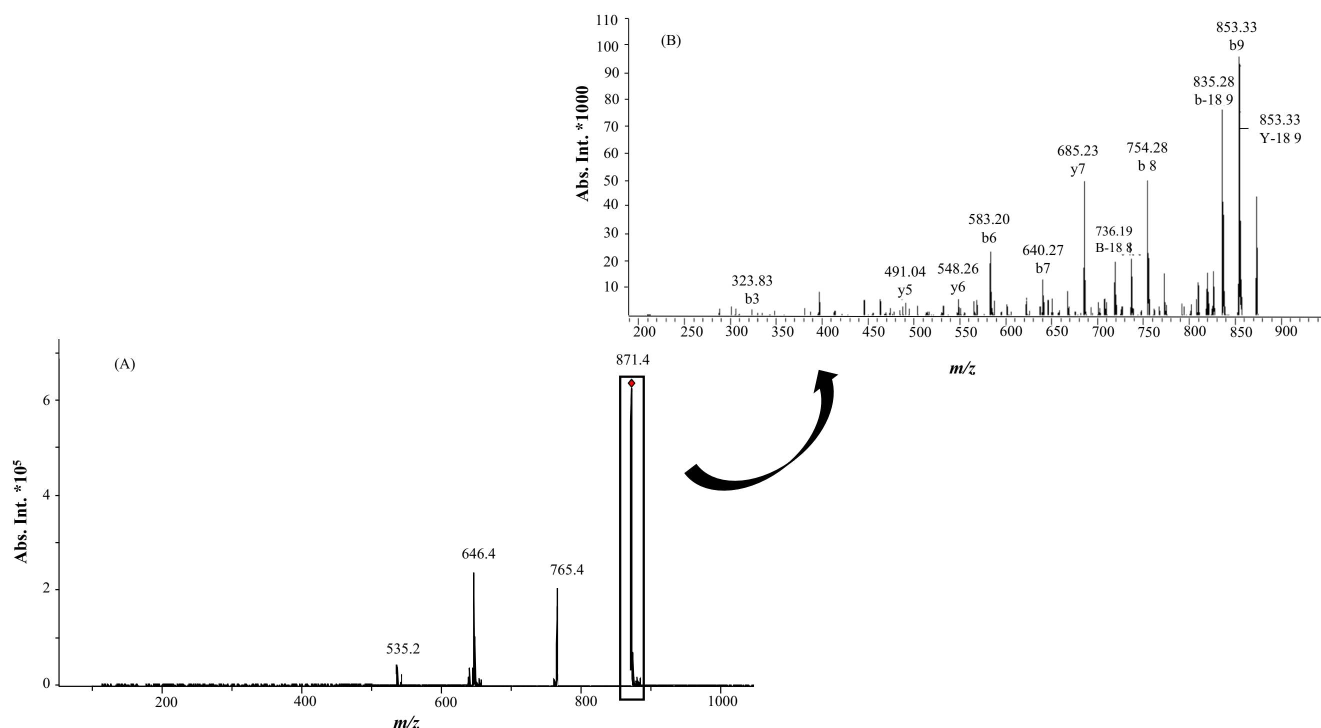

Fig. 3. Identification of potential quinoa bioactive peptides. (A) LC-MS spectrum and (B) LC-MS/MS spectrum of single-charged ion with m/z 871.40. Following sequence interpretation and database searching, the MS/MS spectrum of ion with m/z 871.40 was matched to peptide GEHGSDGNV, corresponding to f (193–201) of 11S seed storage globulin B. Peptide sequence is displayed with ion fragments observed in the spectrum.

Table 4
Inhibitory activity of dipeptidyl peptidase-IV (DPP-IV), α-amylase and α-glucosidase (expressed as% of inhibition at two selected concentrations) of synthetic peptides corresponding to sequences identified by HPLC-MS/MS in the fraction F-2 collected from quinoa protein concentrate digested with pepsin for 120 min and pancreatin for 60 min (digest ID60).

Peptide	Peptide concentration (μM)	Enzyme inhibitory activity (%)		
		DPP-IV[a]	α-amylase[a]	α-glucosidase[a]
IQAEGGLT	250	17.05 ± 0.06	n.d [b]	55.85 ± 0.26
	80	7.40 ± 0.50	n.d [b]	28.62 ± 0.21
DKDYPK	250	n.d.[b]	n.d.[b]	22.16 ± 0.61
	80	n.d [b]	n.d [b]	8.57 ± 0.28
GEHGSDGNV	250	n.d [b]	6.86 ± 0.16	30.84 ± 0.69
	80	n.d [b]	2.33 ± 0.05	12.80 ± 0.36

[a] Values are the mean ± standard deviation.
[b] n.d. no inhibition observed.

enzyme inhibition at 228.03 μM. This result indicates that when mixed, the effects of peptides mixture were higher than those shown by individual peptides, thus suggesting possible interactions among peptides. Moreover, these peptides in combination with those that have not been identified might be determinant on the DPP-IV inhibitory properties shown by fraction F-2.

The mechanism of action proposed for the α-amylase inhibitory capacity of a peptide is related to its ability to form a sliding barrier by establishing a hydrogen bonding with the residues of the active/substrate-binding region (Siow & Gan, 2016; Siow & Gan, 2017). The α-amylase has a number of aromatic residues, which lie within the substrate-binding pocket that could interact directly with the substrate. These residues have been found likely bound by the peptides, especially by their aromatic residues (phenylalanine, tryptophan, and tyrosine). Hence, the aromatic-aromatic interactions between the peptide and enzyme residues arising from hydrogen bonds, electrostatic and Van der Waals interactions, seems to be critically implicated in the inhibitory action of peptides towards the α-amylase (Siow, Lim, & Gan, 2017). Peptide IQAEGGLT does not present any aromatic residue, and only one tyrosine is present in peptide DKDYPK, fact that might explain the lack of α-amylase inhibitory activity shown by these two sequences (Table 4). Peptide GEHGSDGNV showed slight α-amylase inhibitory activity with an IC_{20} value of 921.48 μM. This activity could be due to the presence of glycine since this amino acid has been reported to improve the inhibitory properties of peptides (Ngoh & Gan, 2016) although it cannot be discard the potential contribution of other residues which implication has not been studied yet.

In the case of α-glucosidase, Roskar et al. (2015) suggested that sugar-mimetic compounds are better inhibitors although other compounds of phenolic nature, including flavonoids and tannins have also been reported to exert inhibitory properties (Gonçalves, Mateus, & de Freitas, 2011; You, Chen, Wang, Jiang, & Lin, 2012). Moreover, studies carried out in the last decade have demonstrated the great inhibitory potential of food-derived peptides due to their affinity and specificity of action on molecular target (Lacroix & Li-Chan, 2013; Ren et al., 2016; Yu et al., 2011). The exact mechanism by which peptides could inhibit α-glucosidase activity is still unknown, but it has been suggested that they may exert their inhibitory activity by binding to the enzyme's active site

via hydrophobic interactions as it has been reported for other inhibitors (Bharatham, Bharatham, Park, & Lee, 2008). Ren et al. (2016) confirmed the role of hydrophobic amino acids on the α-glucosidase inhibitory properties of peptides. In our study peptide IQAEGGLT, containing three hydrophobic residues, showed potent inhibitory activity towards α-glucosidase. As shown in Table 4, the inhibition percentages were 28.62 ± 0.21 and 55.85 ± 0.26% when this peptide was assayed at concentrations of 80 and 250 μM, respectively. The IC_{50} value calculated was 109.48 μM. In the case of sequences DKDYPK and GEHGSDGNV, the maximum inhibition percentage reached was 22.16 ± 0.61% and 30.84 ± 0.69%, respectively, at a concentration of 250 μM. The lowest effects shown by these peptides could be due to their higher hydrophilic character.

4. Conclusions

The results of the present study demonstrate the role of quinoa proteins as source of DPP-IV, α-amylase and α-glucosidase inhibitory peptides produced under *in vitro* digestion simulating gastrointestinal conditions. After sequential incubation with pepsin and pancreatin, potent inhibitory digests towards the three enzymes were obtained. Fraction containing peptides <5 kDa is the main responsible for the observed effects although the contribution of larger peptides, mainly on the inhibitory activity against DPP-IV and α-amylase, cannot be dismissed. This is the first time that sequences derived from quinoa proteins are identified and characterized for their biological relevance. Three novel peptides derived from 11S seed storage globulin B have shown potential to inhibit enzymes involved in incretin degradation and digestion of dietary carbohydrates. Because of their demonstrated potential as source of bioactive peptides, quinoa proteins may be used as ingredients for functional foods for the prevention and/or management of T2D. However, it would be interesting to investigate the bioavailability and mechanism(s) of action of the released peptides showing anti-diabetic properties. Also, in order to validate the findings of the present study, further research in cell culture and *in vivo* models should be needed.

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Publicación III: **Propiedades quimiopreventivas de péptidos liberados a partir de quinua (*Chenopodium quinoa* Willd.) bajo condiciones de digestión gastrointestinal simulada.**

R. Vilcacundo, B. Miralles, W. Carrillo, B. Hernández-Ledesma. 2017. Chemopreventive properties of peptides released from quinoa (*Chenopodium quinoa* Willd.) protein under simulated gastrointestinal digestion. Food Research International. Enviado para su publicación.

Resumen

Debido a la continua interacción entre el tracto digestivo y los alimentos, los compuestos dietéticos representan una fuente interesante de agentes quimiopreventivos con acción beneficiosa sobre la salud intestinal. En este estudio, se evaluó la influencia de un modelo de digestión *in vitro* estático estandarizado sobre la liberación de péptidos con potencial quimiopreventivo a partir de las proteínas de quinua. Se investigó la actividad neutralizante de radicales peroxilo y la actividad inhibidora de la viabilidad de células de cáncer de colon de los digeridos gastroduodenales y las fracciones obtenidas mediante ultrafiltración. Los mayores efectos fueron observados en los digeridos obtenidos durante la fase intestinal, siendo la fracción que contenía los péptidos de menor tamaño (< 5 kDa) los responsbales de la actividad antioxidante y aquella que contenía los péptidos más grandes (> 5 kDa) los principales contribuyentes en la actividad citotóxica. En el digerido gastroduodenal obtenido tras 120 minutos de incubación con la pepsina y 60 minutos de incubación con la pancreatina fueron identificados 17 nuevas secuencias peptídicas potencialmente responsables de los efectos quimiopreventivos mostrados por dicho digerido. Por lo tanto, los resultados de este estudio posicionan a las proteínas de quinua como nuevos ingredientes para alimentos funcionales destinados a reducir y/o prevenir aquellos desórdenes asociados al estrés oxidativo, incluido el cáncer.

In vitro chemopreventive properties of peptides released from quinoa (*Chenopodium quinoa* Willd.) protein under simulated gastrointestinal digestion

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Abstract

Because of the continuous and direct interaction between the digestive tract and foods, dietary compounds represent an interesting source of chemopreventive agents for gastrointestinal health. In this study, the influence of a standardized static *in vitro* gastrointestinal digestion model on the release of peptides with chemopreventive potential from quinoa protein was investigated. Gastroduodenal digests and fractions collected by ultrafiltration were evaluated for their *in vitro* oxygen radical absorbance capacity and colon cancer cell viability inhibitory activity. Highest effects were observed in the digests obtained during the intestinal phase, with fraction containing peptides < 5 kDa as the main responsible for the antioxidant activity and peptides > 5 kDa showing the greatest anti-cancer effects. Seventeen potential bioactive peptides derived from quinoa proteins have been identified. These proteins might be utilized as new ingredients in the development of functional foods or nutraceuticals with the aim of reducing oxidative stress-associated diseases, including cancer.

Keywords: quinoa protein; bioactive peptides; simulated gastrointestinal digestion; antioxidant, colon cancer cell viability

1. Introduction

The gastrointestinal tract represents a complex interface system between the body and the external environment. To avoid the passage of dangerous molecules, pathogens, and endotoxins, it has developed several protective mechanisms such as pH modulation, peristalsis, the mucosal gel layer, and the gut-associated lymphoid tissue. In spite of these protective barriers, the gut mucosa is continually exposed to a high concentration of reactive oxygen species (ROS) from endogenous compounds at the luminal surface as well as from exogenous sources and intestinal microbiota (Couto, Goncalves, Catarino, Araújo, Correira-Branco, & Martel, 2012; Graham-Espey, 2013). Excessive ROS levels lead to the formation of oxidative products which cause damage to the epithelial junctions of gut mucosa (Wang et al., 2012). Moreover, ROS are capable of attacking cellular components, such as proteins, lipids, and nucleic acids, causing cytotoxic effects, altered phenotypic patterns, and the uncontrolled transformation of the epithelium. Therefore, growing evidence has demonstrated the influence of oxidative stress on the development of multiple gastrointestinal disorders, including colorectal cancer (Kim, Kim, & Hahm, 2012).

As there is a continuous and direct interaction between the digestive tract and foods, these can exert a positive or negative influence on the gastrointestinal health. Thus, the review and meta-analysis recently published by Vieira and coworkers, as an update of The World Cancer Research Fund International (WCRF) Continuous Update Project (CUP) 2011, has confirmed the protective role of milk and whole grains against colorectal cancer as well as the negative impact on this chronic disorder of red and processed meats and alcohol (Vieira et al., 2017). Dietary compounds represent an interesting source of chemopreventive agents for gastrointestinal health (Moura, Queiroz de Andrade, Farias dos Santos, Pimentel Araújo, & Fonseca Goulart, 2015).

Some plant components possess the capacity to protect the body from malignant cell proliferation caused by free radical-induced oxidative stress (Neri-Numa et al., 2013). Among them, plant-derived peptides are attracting the attention because of their demonstrated health promoting activity without side adverse effects (Chakrabarti, Jahandideh, & Wu, 2014).

Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal consumed by Andean cultures as a staple food. In the last years, the production of quinoa has markedly increased, attracting the interest of the consumers worldwide. This fact has been linked to its agricultural properties, gluten-free nature, and nutritional value. Quinoa seed has been recognized as a very nutritious grain because of the quantity and quality of its proteins (compared to traditional cereals), and the content of fatty acids, dietary fiber, vitamins and minerals. Moreover, multiple phytochemicals present in quinoa provide it a remarkable advantage over other grains in terms of human health (Vilcacundo, & Hernández-Ledesma, 2017). It is known that flavonoids, phenolic acids, and saponins in quinoa contribute to its biological functions. Although some studies have reported a good correlation between the total phenolic content and the antioxidant activity, other authors have suggested the role of non-phenolic compounds such as ascorbic acid, phytic acid, tocopherols, sterols, carotenoids, and ecdysteroids on the antioxidant activity of this seed (Nsimba, Kikuzaki, & Konishi, 2008). Recently, polysaccharides extracted from quinoa with water and alkali have also demonstrated to contribute on the antioxidant activity attributed to this plant (Hu, Zhang, Zou, Fu, Li, & Zhao, 2017).

In addition to their nutritional value, quinoa proteins have been suggested to exert some beneficial effects by themselves, and as a source of bioactive peptides. Quinoa proteins have previously shown 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity when hydrolyzed with Alcalase® (Aluko, & Monu, 2003). More

recently, papain was used to prepare quinoa hydrolyzates with moderate peroxyl-radical scavenging activity (Nongonierma, Le Maux, Dubrulle, Barre, & FitzGerald, 2015). However, to our knowledge, no data about the potential role of quinoa protein as source of dual antioxidant and anti-proliferative peptides during its passage through the gastrointestinal tract are available. With this background, we found appropriate to examine if digestion of quinoa protein might release protein fragments with a positive role on the free-radical-induced oxidative stress and yet protect from malignant cell proliferation. As an attempt to follow internationally accepted digestion conditions, the harmonized *in vitro* digestion protocol developed by the INFOGEST Cost Action (Minekus et al. 2014) was employed. Gastroduodenal digests and chromatographic fractions were assayed by their *in vitro* radical scavenging activity and colon cancer cell viability inhibitory activity. Peptides potentially responsible for the observed effects were identified.

2. Materials and Methods

2.1. Materials

Pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas (EC 232-468-9), porcine bile extract, Pefabloc® SC, fluorescein disodium (FL), dimethylsulfoxide (DMSO), and 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2’-azobis (2-methylpropionamide)-dihydrochloride (AAPH) and 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Aldrich (Milwaukee, WI, USA). The rest of chemicals used were of HPLC grade.

2.2. Obtention of quinoa protein concentrate

The quinoa protein concentrate (QPC) was prepared following the protocol of Toapanta, Carpio, Vilcacundo, & Carrillo (2016) with slight modifications. Quinoa flour (Mascorona, Ambato, Ecuador) was suspended in water (1:10, w/v), and its pH was adjusted to 8.0 with 2 M NaOH. The suspension was stirred for 1 h, and centrifuged at 4,500 x *g* for 30 min at 25 ºC. After adjusting the pH of the supernatant to 4.0 with 2 N HCl, it was centrifuged at 4,500 x *g* during 20 min at 4 ºC. The pellet was dissolved in a small volume of water, neutralized with 0.1 M NaOH, lyophilized, and kept at -20 ºC until further analysis. The protein content of QPC was determined by Kjeldahl method.

2.3. In vitro simulation of gastrointestinal digestion of quinoa protein concentrate

QPC was digested following the harmonized protocol (Minekus et al., 2014). Briefly, QPC was dissolved in water (520 mg/5 mL), and the mixture was diluted with simulated gastric fluid containing pepsin (2000 U/mL of digest) at a ratio of 50:50 (v/v). Digestion was performed at 37 °C in an orbital shaker at 150 rpm. Samples were withdrawn at the beginning (Q0) and after 120 min of gastric digestion (QG120), stopping the reaction by adjusting the pH at 7.0 with 1 M NaOH, and snap freezing in liquid nitrogen. Gastric phase was mixed (ratio 50:50, v/v) with simulated intestinal fluid containing pancreatin (100 U trypsin activity/mL of digest) and porcine bile extract (10 mM in the final mixture). Samples were withdrawn after 60 (QD60) and 120 min (QD120) of intestinal digestion, stopping the reaction with Pefabloc® SC (5 mM) and snap freezing. Digestion was performed in duplicate. A digestion blank containing the mixture of enzymes used in digestions at the same concentration without QPC was prepared.

Digests Q0, QG120, QD60, and QD120 were subjected to ultrafiltration through a hydrophilic 5000 Da cutoff membrane (Agilent Technologies, Inc., Waldbronn, Germany). Fractions < 5 kDa and > 5 kDa were frozen dried and kept at -20 °C until use. The peptide content of digests and fractions was determined by the bicinchoninic acid method (BCA) (Pierce, Rockford, IL, USA), using bovine serum albumin as standard protein.

2.4. SDS-PAGE

The protein and peptide pattern of QPC and gastric and gastroduodenal digests was evaluated by SDS-PAGE. Samples (1 mg protein/mL) were dissolved in sample buffer composed by Tris-HCl (0.05 M, pH 6.8), SDS (1.6%, w:v), glycerol (8%, v:v), β-mercaptoethanol (2%, v:v) and bromophenol blue indicator (0.002%, w:v), and heated at 95 °C for 5 min. They were loaded into 12% Bis-Trispolyacrilamide gels (Criterion_XT, Bio-Rad, Hercules, CA, USA) and electrophoretic separation was carried out at 100 V for 5 min and then at 150 V, using the XT MES running buffer (Bio-Rad) in the criterion cell (Bio-Rad). The molecular weight marker (Precision Plus ProteinTM Unstainend standard, Bio-Rad) containing ten Strep-tagged recombinant proteins (10–250 kDa) was used. Gels were stained with Instant Coomasie Blue (Expedeon, Swavesey, UK) and images were taken with a Molecular Imager Versa-DocTM MP 5000 system (Bio-Rad).

2.5. Measurement of chemopreventive activity

2.5.1. Oxygen radical absorbance capacity (ORAC)

An oxygen radical absorbance capacity (ORAC)-FL assay was used based on the protocol optimized by Hernández-Ledesma, Dávalos, Bartolomé, and Amigo, (2005).

Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4). The final assay mixture (200 μL) contained FL (70 nM), AAPH (14 μM), and antioxidant Trolox (0.2-1.6 nmol) or sample (at different concentrations). Fluorescence was recorded during 137 min in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters. The equipment was controlled by the FLUOstar Control ver. 1.32 R2 software for fluorescence measurement. Three independent runs were performed for each sample. Final ORAC-FL value was expressed as μmol Trolox equivalents per mg protein or peptide.

2.5.2. Colon cancer cell viability inhibitory activity

Human colorectal cancer cell lines (Caco-2, HT-29, and HCT-116) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). HT-29 and HCT-116 cells were grown in McCoy´s medium (Lonza Group Ltd., Basel, Switzerland), and Caco-2 cells in Dulbecco´s Modified Eagle Medium (DMEM, Biowest, Nuaillé, France). Media were supplemented with 10% (v:v) fetal bovine serum and 1% (v:v) penicillin/streptomycin/amphotericin B solution (Biowest). A non-essential amino acid solution (Lonza Group Ltd.) was also added to DMEM medium (1%, v:v) for the culture of Caco-2 cells. The cells were maintained at 37 °C in an incubator under a 5 % CO_2/95 % air at constant humidity. Culture medium was changed every two days, and cells were kept sub-confluent by using trypsin/EDTA (Lonza Group Ltd.).

The MTT assay was performed to evaluate the effect of QPC digests and fractions on the cancer cells viability. Cells were seeded in 96-well plates (Costar, Corning, NY, USA) at a density of 5×10^4 cells/well. After 24 h incubation, cells were

treated with QPC digests, fractions or blanks at concentrations ranging from 4 to 0.031 mg/mL for 24 h. Then, culture medium was removed, and cells were washed with phosphate buffer saline (Lonza Group Ltd.) and incubated for 2 h with a MTT solution (0.5 mg/mL, final concentration). The supernatants were discarded, and insoluble formazan crystals were dissolved in DMSO:ethanol (1:1, v:v). After gently mixing, the absorbance was measured at 570 nm in a FLUOstar OPTIMA plate reader (BMG Labtech). Experiments were carried out in triplicate. Results were expressed as IC_{50} or protein/peptide concentration needed to inhibit 50% cell viability.

2.6. Fractionation of the quinoa protein digest by semi-preparative RP-HPLC

QD60, at a concentration of 10 mg/mL, was separated as reported in Vilcacundo, Martínez-Villaluenga, & Hernández-Ledesma (2017) using a Nova-Pak HR C18 (300mm × 7.8mm internal diameter, Waters Corp., Milford, MA, USA) column, in a Waters 600 HPLC equipped with two pumps (module delta 600), a pump controller (module 600), an autosampler (module 717), and a diode array detector (module 996) in combination with an automatic fractions collector (module II). Three fractions were collected from 45-50 runs, pooled, lyophilized, and stored at -20 °C until further analyses. The peptide content of fractions was determined by the BCA method.

2.7. Characterization of fractions from quinoa protein concentrate digest

Amino acids of fractions collected from QD60 were analyzed in duplicate by cation exchange chromatography using a Biochrom 30 series Amino Acid Analyzer (Biochrom, Cambridge, USA) after automatic pre-column derivatization of samples with Ortho-phthalaldehyde (OPA). Detection was performed by monitoring the

absorbance at 440 nm. The samples were previously hydrolyzed with 6 N HCl for 21 h at 110 °C.

To analyze the peptide mass distribution, MALDI-TOF mass spectrometry (MS) was performed on a Bruker Autoflex Speed® (Bruker Daltonics GmbH, Bremen, Germany) as previously reported by Lozano-Ojalvo, Molina, & Lopez-Fandiño (2016). Samples were spotted on a Bruker Anchorchip target with α-CHCA matrix in acetonitrile/water (30:70) containing 0.1% trifluoroacetic acid. Mass spectra were acquired in positive reflectron mode by accumulating 1000 laser pulses on average. Calibration was performed with the Peptide Calibration Standard I (Bruker Daltonics).

2.8. Peptide identification by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

RP-HPLC coupled to tandem MS (RP-HPLC-MS/MS) analysis of collected fractions (0.5 mg protein/mL) was performed as previously reported (Sanchón et al., 2018). The acquired MS/MS spectra were interpreted using BioTools (version 3.1; Bruker Daltonics). Available quinoa protein sequences were included in a homemade database used for peptide sequencing with MASCOT v2.4 software (Matrix Science, Boston, MA, USA). No specific enzyme cleavage was used. Peptide mass tolerance was set to 0.1 % and 0.5 Da for MS and MS/MS analysis, respectively. Each identification spectrum was manually revised, regardless of its score.

2.9. Statistical analysis

Data represent the mean and standard deviation (SD) of three independent experiments (n = 3). Data were subjected to one-way analysis of variance (ANOVA) to compare experimental values using Statgraphics 5.0 (Statistical Graphics Corporation,

Rockville, MD, USA). Comparison between groups was performed using a Duncan's multiple-range test, and differences were considered significant at $P \leq 0.05$.

3. Results and Discussion

3.1. Antioxidant and colon cancer cell inhibitory activity of quinoa protein concentrate over the course of simulated gastrointestinal digestion

Non-digested QPC (Q0), and gastric (QG120) and gastroduodenal digests (QD60 and QD120) were analyzed by their ability to scavenge oxygen radicals. As shown in Figure 1A, undigested protein showed an ORAC value of 0.42 ± 0.03 µmol Trolox equivalents/mg protein that was of the same order than that reported by Nongonierma et al. (2015) for quinoa protein used as control for their determinations (0.26 ± 0.07 µmol Trolox equivalents/mg sample). This result suggests that amino acids or peptides present in QPC before hydrolysis might exert a slight antioxidant activity. Over the course of the *in vitro* simulated digestion, the activity significantly increased. As it was observed by SDS-PAGE (Figure S1), incubation with pepsin resulted in a partial hydrolysis of quinoa proteins whereas sequential incubation with pepsin and pancreatin provoked the complete degradation of the proteins releasing small peptides that leached out from the gel. Peptides released during gastric phase were responsible for the ORAC value determined in QG120 (1.03 ± 0.06 µmol Trolox equivalents/mg protein). The ORAC values of gastroduodenal digests obtained after incubation with pancreatin for 60 min (2.22 ± 0.07 µmol Trolox equivalents/mg protein) and 120 min (2.39 ± 0.16 µmol Trolox equivalents/mg protein) were not significantly different ($P < 0.05$), indicating that peptides released during the first 60 minutes of the duodenal phase were very active as radical scavengers and resisted further hydrolysis by pancreatin, maintaining their activity until the end of digestion. The ORAC values were higher than

that determined by Nongonierma et al. (2015) for quinoa protein hydrolyzed by papain (0.50 μmol Trolox equivalents/mg sample), suggesting the important role of gastrointestinal digestion on the release of antioxidant peptides from this plant protein. Previous studies had also described the key impact of gastrointestinal digestion on the release of bioactive peptides from parent proteins as well as on the modification or breakdown of peptides with antioxidant properties (Gallegos Tintoré et al., 2015; Gallego, Mora, Hayes, Reig, & Toldrá, 2017). Orsini Delgado, Tironi, & Añón, (2011) determined the antioxidant capacity of amaranth proteins digested under *in vitro* gastrointestinal conditions. The ORAC values obtained by these authors (ranged from 0.80 to 1.16 μmol Trolox equivalents/mg protein) were lower than those found in our study. The differing digestion conditions might have caused the release of less active protein hydrolysis products. Even though, the great difference in ORAC values suggests that, in spite of the similarity between amaranth and quinoa as pseudocereals, quinoa protein is shown as a better source of antioxidant peptides under gastrointestinal digestion conditions.

In order to determine the influence of the molecular weight of peptides on the antioxidant activity of quinoa protein digests, Q0, QG120, QD60, and QD120 were separated into two molecular weight peptide fractions, > 5 kDa and < 5 kDa whose antioxidant capacity was measured (Figure 1B). ORAC values of fractions containing short peptides were higher than those of fractions comprising large peptides. Short peptides contained in fraction < 5 kDa obtained from quinoa protein hydrolyzed with Alcalase® had also demonstrated to exert higher DPPH radical scavenging activity than peptides contained in fraction > 10 kDa (Aluko & Monu, 2003). Absence of activity was reported for the original hydrolyzate before it as passed through ultrafiltration membranes. Other studies have also described the radical scavenging activity to be

associated with low molecular weight peptides (Roblet et al., 2012). Fraction < 5 kDa obtained from gastroduodenal digest QD60 showed the most potent oxygen radical scavenging capacity with an ORAC value of 2.72 ± 0.10 µmol Trolox equivalents/mg protein (Figure 1B). A slight antioxidant activity was measured in the fraction > 5 kDa from QG120, QD60, and QD120 with ORAC values in the range 0.6-0.7 µmol Trolox equivalents/mg protein. This result might be explained by the ultrafiltration method carried out to separate both fractions that could allow the passage of low molecular weight active peptides to fraction > 5 kDa. Moreover, the antioxidant capacity is not only related to the size of peptides but also to their amino acid composition, structure, and hydrophobic character, which determine the mechanism and efficiency of the antioxidants (Gallego et al., 2017).

Cytotoxic activity against cancer cells was considered to be the effect produced when the hydrolyzate was added to the culture medium after the cells had been growing for 24 h (Alemán, Pérez-Santín, Bordenave-Juchereau, Arnaudin, Gómez-Guillén, & Montero, 2011). In our study, the potential cytotoxic activity in the gastrointestinal tract of QPC and its digests was evaluated by the MTT protocol. A first screening study using Caco-2 cells was carried out with Q0, QG120, QD60, and QD120 digests. To evaluate the effect of digestive enzymes and bile salts on cell viability, digestion blanks without QPC were also assayed (Figure 2A). Q0 showed no inhibitory effect at the highest concentration used (4.0 mg protein/mL). However, QG120 showed anti-cancer ability against cancer cells in a dose dependent manner. The maximum inhibitory activity observed was 81.2 % when Caco-2 cells were exposed to 4 mg protein/mL of QG120 while its corresponding digestion blank did not show any effect at this concentration, suggesting that peptides released by the action of pepsin could be responsible for the observed effects. The IC_{50} value of QG120 digest was 0.843 ± 0.001

mg protein/mL. The cell viability inhibitory activity of gastroduodenal digests significantly increased in comparison with that shown by gastric digest. Thus, the IC_{50} values of QD60 and QD120 were 0.191 ± 0.003 mg protein/mL and 0.180 ± 0.001 mg protein/mL, respectively. This result indicates that more potent peptides inhibiting Caco-2 cell viability were released by pancreatin, although the contribution of this enzyme and bile salts on the activity cannot be discarded. The corresponding digest blanks showed an IC_{50} value of 1.241 ± 0.040 mg/mL (Figure 2A). Since any significant difference ($P < 0.05$) was observed between digests obtained after 60 and 120 min incubation with pancreatin, QD60 was selected to evaluate its activity against HT-29 and HCT-116 cells. The IC_{50} values were 0.212 ± 0.002 mg protein/mL and 0.208 ± 0.002 mg protein/mL, respectively. Although these values were significantly similar to that shown against Caco-2 cells, the sensitivity of HT-29 and HCT-116 cells to the enzyme and bile salts was inferior and thus, the IC_{50} values of the digestion blanks for these two cell lines were 0.745 ± 0.012 and 0.759 ± 0.007 mg protein/mL, respectively. This fact suggests that peptides released during the duodenal phase were more potent inhibiting the viability of Caco-2 cells. The highest sensitivity of Caco-2 cells in comparison to HT-29 and HCT-116 cells has also been recently reported for the gastroduodenal digest obtained from germinated soybean proteins (González-Montoya, Hernández-Ledesma, Silván, Mora-Escobedo, & Martínez-Villaluenga, 2017). Differences in cytotoxic activity among cell lines might be due to differences in cell membrane composition, fluidity and surface area (Leuschner, & Hansel, 2004).

Fractions > 5 kDa and < 5 kDa obtained from QD60 were also assayed for their cytotoxic effects against three colon cancer cells (Figure 2). Between ultrafiltration fractions, > 5 kDa peptides showed higher potency (IC_{50} = 0.239 ± 0.001 mg protein/mL) to inhibit Caco-2 cell viability than < 5 kDa peptides (IC_{50} = 0.676 ± 0.007

mg protein/mL). Similar behavior was observed against HT-29 and HCT-116 cells, with IC_{50} values of 0.085 ± 0.003 and 0.176 ± 0.000 mg protein/mL, respectively, for fraction > 5 kDa, and 0.781 ± 0.009 and 0.928 ± 0.012 mg protein/mL, respectively, for fraction < 5 kDa. Digestive enzymes retained in fraction > 5 kDa, and bile salts or enzyme inhibitor contained in Pefabloc® SC that passed through the membrane filter appearing in fraction < 5 kDa could be responsible for the cytotoxic effects shown by fractions collected from the digestion blank. All together indicated that quinoa peptides with higher molecular weight (> 5 kDa) were more effective inhibiting colon cancer cell proliferation than smaller molecular weight peptides (< 5 kDa). This is in agreement with Rayaprolu, Hettiarachchy, Chen, Kannan, & Mauromostakos, (2013) who found fraction 10-50 kDa obtained from soybean meal protein as the most active inhibiting human colon, liver, and lung cells viability. González-Montoya et al. (2017) also reported the most potent activity for high molecular fractions collected from germinated soybean digests. However, Kannan, Hettiarachchy, Johnson, & Nannapaneni, (2008) found the < 5 kDa fraction of rice bran peptide hydrolyzates to have higher anti-cancer activity against HepG-2 and Caco-2 cells than both the 5-10 and >10 kDa fractions. Although the greater molecular mobility and diffusivity of low molecular weight peptides has been considered to improve interactions with cancer cell components and enhance antiproliferative activity (Jumeri, & Kim, 2011), in our study, other aspects such as the amino acid composition and the peptide hydrophobicity could also have a positive impact on the activity of > 5 kDa fraction.

3.2. Characterization of peptide fractions collected by RP-HPLC

QD60 and its fractions > 5 kDa and < 5 kDa were analyzed by RP-HPLC (Figure 3). As it is shown in Figure 3A, the majority of the peptides contained in whole

digest eluted between 5 and 45 minutes. A similar chromatographic pattern was observed for fraction < 5 kDa (Figure 3C), although the intensity of peaks eluting during the first minutes (5-25 minutes) was higher, and that of peaks eluting from 25 to 45 minutes was lower than that observed for the whole digest. The profile of fraction > 5 kDa was slightly different with peaks of lower intensity, although the presence of longer and more hydrophobic peptides at the final region of chromatogram was visible (Figure 3B). Since fractions > 5 kDa and < 5 kDa were primarily responsible for cell viability, and antioxidant activities, respectively, the whole digest QD60 was selected to separate peptide fractions. Three fractions (F-1, F-2, and F-3, Figure 3A) were collected by successive HPLC analysis and assayed for their protein concentration, amino acid composition, molecular weight distribution, and biological activities. The peptide concentration of F-1, F-2, and F-3, as determined by the BCA method, were 38.0, 50.0, and 48.0 % respectively. The lower peptide content of F-1 could be due to the elution of the hydrophilic salts into this fraction at the beginning of the gradient separation. The amino acid composition is shown in Table 1. The analysis conditions did not allow the identification of tryptophan (W) because of its destruction by acid hydrolysis. The total amino acid content of QD60 was 49.22 %, with presence of both essential and non-essential amino acids. The percentage ratio of essential to total amino acids was 31 %, which is necessary for an ideal protein to meet the amino acid requirements. Negatively charged (15.01 %) amino acids were the most abundant followed by hydrophobic (14.77 %), positively charged (8.63 %), branched-chain (7.16 %), and aromatic (4.13 %) amino acids. Total amino acid content of fraction F-1 (67.88 %) and F-2 (86.63 %) were 1.4 and 1.8-fold higher than that determined in the whole digest. Levels of aromatic amino acids phenylalanine (F) and tyrosine (Y) increased with retention time up to 1.1 and 3.6-fold in F-1 and F-2, respectively. Increases in levels of branched chain

and hydrophobic amino acids were also observed in these two fractions in comparison with QD60. In contrast, the content of positively charged amino acids in fraction F-2 was 1.8-fold lower than that of QD60. These results support the fact that residence time on the column is mainly influenced by the peptide hydrophobicity (Girgih, Chibuike, Udenigwe, Hasan, Gill, & Aluko, 2013). However, the observed decreases in hydrophobic and aromatic amino acid levels in fraction F-3 suggest that other aspects such as the peptide size also need to be taken in account. Thus, the analysis of peptide mass distribution showed that peptides with molecular weight lower than 1100 kDa were the most abundant in fraction F-1 (30 % of total amino acids) decreasing this percentage to 23.0 and 16.5 % in fractions F-2 and F-3, respectively (Figure S2). Fraction F-2 showed the most elevated content of peptides with molecular weight higher than 1101 kDa (15 %). Although it was expected that the longest peptides were mainly present in fraction F-3, due to the limitations of the method, it was not possible to confirm this hypothesis.

Three fractions showed antioxidant activity by the ORAC assay (Table 2). Thus, taken as a whole, the separated peptides have a high potential to disrupt reactions that involve peroxyl radicals, although the highest values corresponded to fractions F-1 () and F-2 (), without significant differences between them. An apparent molecular weight of the whole fraction was determined to express the values in molarity. Thus, F-1, F-2, and F-3 showed 1.50 ± 0.14, 1.67 ± 0.11, and 0.78 µmol Trolox equivalents/µmol peptide, respectively. This is consistent with studies where high radical scavenging activities for the protein hydrolyzates or peptides are usually associated with high hydrophobic and/or aromatic amino acid content (Rajapakse, Mendis, Byun, & Kim, 2005). The hydrophobic amino acids represented 21.71 % and 38.87 % of the total amino acids in fraction F-1 and F-2 (Table 1). This might increase the solubility of the

peptides in lipids, facilitating a better interaction with free radicals. Moreover, the aromatic amino acids might donate protons to electron deficient radicals, maintaining their stability. Their content in F-1 and F-2 were also important, 4.56 % and 15.05 %, respectively. Therefore, the amino acid composition of fractions collected from QD60 was compatible with the presence of peptides with antioxidant capacity. Low molecular weight peptides contained in fractions F-1 and F-2 could also contribute on the high ORAC values obtained. The majority of antioxidant peptides derived from food sources have molecular weight between 500 and 1800 Da (Samaranayaka & Li-Chan, 2011). Their increased ability to interact with free radicals when compared to bigger peptides make small size peptides good oxygen radical scavengers (Onuh, Girgih, Aluko, and Aliani, 2014).

The cell viability inhibitory activity against Caco-2, HT-29, and HCT-116 cells of fractions F-1 to F-3, expressed as IC_{50}, is shown in Table 2. F-1 and F-2 did not show cytotoxic effects on Caco-2 cells at the highest concentration (1 mg/mL) assayed. However, both fractions inhibited viability of HT-29 and HCT-116 cells in a dose-dependent manner, although HT-29 cells showed to be more sensitive to the action of peptides contained in these fractions. Thus, the IC_{50} values shown by F-1 and F-2 against HT-29 cells were 0.600 ± 0.006 mg peptide/mL (0.677 ± 0.007 mmol peptide) and 0.594 ± 0.003 mg peptide/mL (0.544 ± 0.003 mmol peptide), respectively. F-3 was found to be the most potent fraction inhibiting the viability of colon cancer cells. The IC_{50} values calculated against Caco-2, HT-29, and HCT-116 cells were 0.256 ± 0.004, 0.195 ± 0.001, and 0.193 ± 0.003 mg peptide/mL, respectively. Using the apparent molecular weight of the whole fraction, the IC_{50} values expressed as mmol peptide were 0.251 ± 0.004, 0.191 ± 0.001, and 0.189 ± 0.003 for Caco-2, HT-29, and HCT-116 cells, respectively. These values were similar or slightly inferior to those shown by the whole

digest, suggesting the peptides contained in F-3 were the main responsible for the anti-cancer effects at intestinal level shown by QPC after its simulated gastrointestinal digestion. These effects cannot be totally attributed to peptides containing aromatic and/or hydrophobic amino acids because the contents of these residues were lower in F-3 in comparison with the other two fractions. As it has been previously reported, the antiproliferative activity could be related to the presence of specific peptides exerting a direct cytotoxicity on cancer cells. Thus, peptide EGRPR, extracted from rice bran, was demonstrated to cause the 85 % inhibition of the growth of colon cancer cells Caco-2 and HCT-116 at a dose of 600-700 µg/mL (Kannan, Hettiarachchya, Lay, & Liyanage, 2010).

3.3. Identification of potential chemopreventive peptides

In order to identify the peptides present in the chromatographic fractions, they were analyzed by LC-MS/MS. With this analysis, many peptide compounds were detected although only seventeen fragments matched sequenced proteins from quinoa, four of them contained in F-1, five in F-2, and eight in F-3 (Table 3). Seven of these peptides had been previously identified by Vilcacundo et al. (2017). The majority of identified peptides (13 of 17) corresponded to fragments of 11S globulin, or of its subunits A and B. According to the literature, 11S globulin, called chenopodin, is the major seed storage protein of quinoa seeds accounting for 37 % of the total protein (Fairbanks, Burgener, Robison, Andersen, & Ballon, 1990). Two fragments found in fraction F-3 corresponded to the sequence of maturase K, one corresponded to betaine aldehyde dehydrogenase, and one fragment belonged to starch synthase, chloroplastic/amyloplastic. Chenopodin peptides identified in our study were rich in glycine (G), glutamic acid (E), alanine (A), and aspartic acid (D). Typical of 11S storage globulins, chenopodin has been found to be high in glutamine/glutamic acid

(Q/E), asparagine/aspartic acid (N/D), arginine (R), serine (S), leucine (L), and glycine (G) (Brinegar, & Goundan, 1993). Amino acids tryptophan (W) (ORAC-FL value of 4.65 µmol Trolox/µmol of amino acid), tyrosine (Y) (1.57 µmol Trolox/µmol of amino acid), and methionine (M) (1.13 µmol Trolox/µmol of amino acid) have been reported as the main contributors on the peroxyl radical of peptides, with a slight role of residues cysteine (C), histidine (H), and phenylalanine (F) (Hernández-Ledesma et al., 2005). Therefore, quinoa-derived peptides LWREGM (F-1), DKDYPK (F-2), and DVYSPEAG, IFQEYI, and RELGEWGI (F-3) might be responsible for the antioxidant effects observed. As potential protecting agents against oxidative stress, these peptides might also act as anti-cancer compounds, since it has been demonstrated that inhibition of oxidative stress inhibition leads to reduce genetic alteration such as mutation and chromosomal rearrangements which play a vital role in the initiation of carcinogenesis (Jumeri, & Kim, 2011). Some previous reports have proposed that antioxidant peptides have the potential to prevent and treat diseases associated with active oxygen species, especially some forms of cancer (Sheih, Fang, Wu, & Lin, 2010; You, Zhao, Liu, & Regenstein, 2011).

4. Conclusions

The results obtained in the present study show quinoa proteins as a potential source of chemopreventive peptides which are released by an *in vitro* treatment simulating physiological digestion conditions. After sequential incubation with pepsin and pancreatin, potent antioxidant and colon cancer cell viability inhibitory digests were obtained. Fraction containing peptides < 5 kDa was the main responsible for the radical scavenging activity while peptides > 5 kDa showed greater anti-cancer effects. Seventeen potential bioactive peptides derived from 11S globulin and other quinoa

proteins have been identified. They might be utilized as a new ingredient in the development of functional foods or nutraceuticals with the aim of reducing oxidative stress-associated diseases, including cancer. Further work is needed to investigate the bioavailability and mechanism(s) of action of released chemopreventive peptides, and to elucidate their real relevance in the human body.

Acknowledgments

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Figure captions

Figure 1. Oxygen radical absorbance capacity (ORAC), expressed as μmol Trolox equivalents/mg protein, of (A) (■) gastric and gastroduodenal digests from quinoa protein concentrate: Q0: digest at starting point; QG120: gastric digest after 120 min incubation with pepsin; QD60: gastroduodenal digest after 120 min incubation with pepsin and 60 min incubation with pancreatin; QD120: gastroduodenal digest after 120 min incubation with pepsin and 60 min incubation with pancreatin. (B) ■) fraction > 5 kDa and □) fraction < 5 kDa obtained from gastric and gastroduodenal digests. Different lowercase letters for each graph indicate significant differences among samples ($P < 0.05$, Duncan test)

Figure 2. (A) Colon cancer cell viability inhibitory activity against Caco-2 cells, expressed as IC_{50} (mg protein/mL) of (□) digestion blanks corresponding to the gastric digest after 120 min incubation with pepsin (QG120), and gastroduodenal digests after 60 min (QD60) and 120 min (QD120) incubation with pancreatin and (■) digests QG120, QD60, and QD120; (B) Colon cancer cell (Caco-2, HT-29, and HCT-116) viability inhibitory activity, expressed as IC_{50} (mg protein/mL) of (□) fraction > 5 kDa obtained from the digestion blank corresponding to QD60, (■) fraction > 5 kDa obtained from QD60, (□) fraction < 5 kDa obtained from the digestion blank corresponding to QD60, and (■) fraction < 5 kDa obtained from QD60. n.d.: inhibition no detected at the highest concentration used (4.0 mg/mL). Different lowercase letters indicate significant differences among samples ($P < 0.05$, Duncan test)

Figure 3. UV-chromatograms of (A) gastroduodenal digest after 120 min incubation with pepsin and 60 min incubation with pancreatin (QD60), and the peptide fractions (B) > 5 kDa and (C) < 5 kDa obtained by ultrafiltration from QD60.

Table 1. Amino acid composition (%) of the gastroduodenal digest of quinoa protein concentrate after 120 min incubation with pepsin and 60 min incubation with pancreatin (QD60) and its fractions F-1, F-2, and F-3 collected by RP-HPLC

Aminoacid	QD60	F-1	F-2	F-3
Aspartic acid (D)	6.44 ± 0.03	7.76 ± 0.46	11.14 ± 0.40	8.23 ± 0.47
Threonine (T)	1.69 ± 0.02	2.41 ± 0.17	2.97 ± 0.22	1.21 ± 0.09
Serine (S)	2.95 ± 0.11	3.87 ± 0.26	3.93 ± 0.16	2.15 ± 0.04
Glutamic acid (E)	8.57 ± 0.10	14.07 ± 1.20	14.65 ± 0.18	5.41 ± 0.39
Glycine (G)	4.15 ± 0.06	2.95 ± 0.34	4.46 ± 0.05	5.44 ± 0.67
Alanine (A)	2.24 ± 0.00	2.98 ± 0.15	3.25 ± 0.04	1.25 ± 0.02
Cysteine (C)	0.58 ± 0.04	0.94 ± 0.15	1.93 ± 0.60	1.25 ± 0.03
Valine (V)	2.09 ± 0.01	3.26 ± 0.30	3.70 ± 0.16	1.86 ± 0.05
Methionine (M)	0.66 ± 0.02	1.09 ± 0.03	1.26 ± 0.09	0.50 ± 0.03
Isoleucine (I)	3.57 ± 0.02	6.33 ± 0.36	9.76 ± 0.46	3.08 ± 0.34
Leucine (L)	1.50 ± 0.00	2.55 ± 0.09	3.92 ± 0.39	1.79 ± 0.17
Tyrosine (Y)	1.71 ± 0.00	4.36 ± 0.42	2.85 ± 0.27	0.78 ± 0.04
Phenylalanine (F)	2.42 ± 0.01	0.20 ± 0.03	12.20 ± 0.47	1.93 ± 0.25
Histidine (H)	1.21 ± 0.01	1.87 ± 0.15	1.78 ± 0.18	0.67 ± 0.02
Lysine (K)	3.35 ± 0.07	4.84 ± 0.32	1.66 ± 0.16	0.83 ± 0.01
Arginine (R)	4.07 ± 0.05	6.07 ± 0.84	1.49 ± 0.15	0.82 ± 0.00
Proline (P)	2.02 ± 0.04	2.33 ± 0.21	5.68 ± 0.40	3.33 ± 0.20
Aromatic[a]	4.13	4.56	15.05	2.71
Branched chain[b]	7.16	12.14	17.38	6.73
Hydrophobic[c]	14.77	21.71	38.87	12.44
Positively charged[d]	8.63	12.78	4.93	2.32
Negatively charged[e]	15.01	21.83	25.79	13.64
TOTAL	**49.22**	**67.88**	**86.63**	**40.53**

[a]: Phenylalanine + Tyrosine + Tryptophan
[b]: Leucine + Isoleucine + Valine
[c]: Alanine + Valine + Leucine + Isoleucine + Tyrosine + Phenylalanine + Tryptophan + Methionine + Cysteine
[d]: Arginine + Histidine + Lysine
[e]: Aspartic acid + Glutamic acid

Table 2. Antioxidant and colon cancer cells viability inhibitory activity of fractions obtained by preparative RP-HPLC from gastroduodenal digest of quinoa protein concentrate after 120 min incubation with pepsin and 60 min incubation with pancreatin (QD60). The results of antioxidant activity (ORAC assay) were expressed as μmol Trolox equivalents/mg protein. Cell viability was evaluated by the MTT assay in human colon cancer Caco-2, HT-29, and HCT-116 cells, and results were expressed as IC_{50} (mg protein/mL)

Fraction	Antioxidant activity (ORAC, μmol Trolox equivs/mg peptide)	Cell viability inhibitory activity (IC_{50}, mg peptide/mL)		
		Caco-2	HT-29	HCT-116
F-1	1.70 ± 0.16^a	n.d.$^+$	0.600 ± 0.006^b	0.746 ± 0.001^d
F-2	1.54 ± 0.10^a	n.d.$^+$	0.594 ± 0.003^b	0.807 ± 0.008^e
F-3	0.76 ± 0.06^b	0.256 ± 0.004^a	0.195 ± 0.001^c	0.193 ± 0.003^c

n.d.$^+$: No inhibitory effect observed at the highest concentration used (1 mg/mL)
$^{a\text{-}c}$: Different lowercase letters in each biological activity (antioxidant and cell viability inhibitory activity) indicate significant differences among samples ($P < 0.05$, Duncan test)

Table 3. Peptides identified by LC-MS/MS in the fractions F-1, F-2, and F-3 collected by RP-HPLC from gastroduodenal digest of quinoa protein concentrate after 120 min incubation with pepsin and 60 min with pancreatin (QD60)

Fraction	Observed mass	Calculated mass	Source protein	NCBI Accession number	Fragment	Sequence
F-1	786.32	786.47	11S Globulin A	ABI94735.1	f(76-81)	IRRTIE
	790.32	790.38	11S Globulin A	ABI94735.1	f(124-129)	LWREGM
	686.32	686.36	11S Globulin	AAS67037.1	f(51-57)	IQAEGGL
	627.32	627.33	11S Globulin	AAS67037.1	f(198-203)	AGKPQQ
F-2	787.40	787.41	11S Globulin B	ABI94736.1	f(26-33)	IQAEGGLT
	764.40	764.37	11S Globulin B	ABI94736.1	f(163-168)	DKDYPK
	870.40	870.35	11S Globulin B	ABI94736.1	f(193-201)	GEHGSDGNV
	729.39	729.33	11S Globulin	AAS67037.1	f(166-172)	NSGNEPL
	865.39	865.34	11S Globulin	AAS67037.1	f(404-411)	QAGEEGFE
F-3	875.41	875.35	11S Globulin	AAS67037.1	f(303-310)	SENIDDPS
	836.44	836.35	11S Globulin	AAS67037.1	f(313-320)	DVYSPEAG
	859.47	859.47	11S Globulin	AAS67037.1	f(318-325)	EAGRLTTL
	685.39	685.39	11S Globulin	AAS67037.1	f(433-438)	IRAMPV
	811.30	811.41	Maturase K	CCI55135.1	f(23-28)	IFQEYI
	758.40	758.40	Maturase K	CCI55135.1	f(224-229)	SFFVFL
	958.50	958.49	Betaine aldehyde dehydrogenase	ALA65446.1	f(469-476)	RELGEWGI
	953.50	953.52	Starch synthase, chloroplastic/amyloplastic	ALB36792.1	f(15-25)	GGLGDVLGGLP

Figure 1

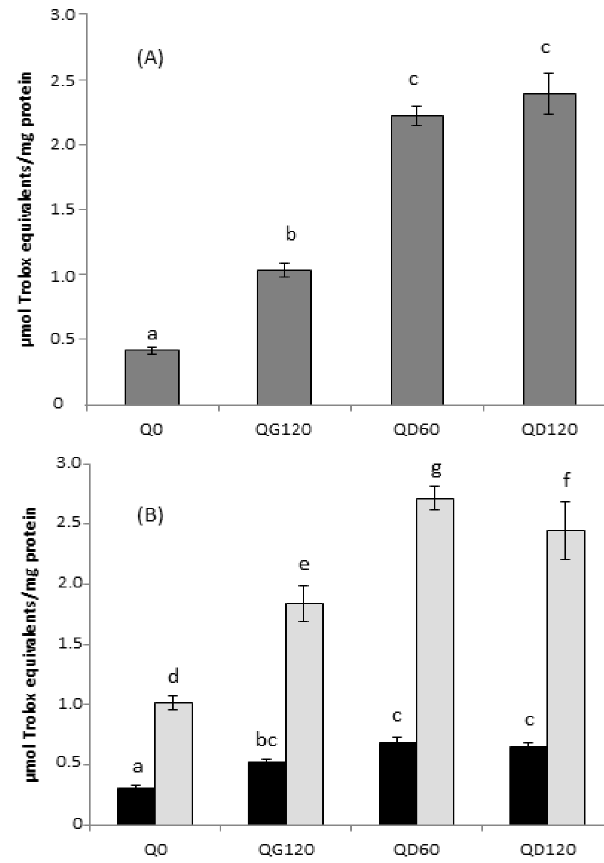

Figure 2

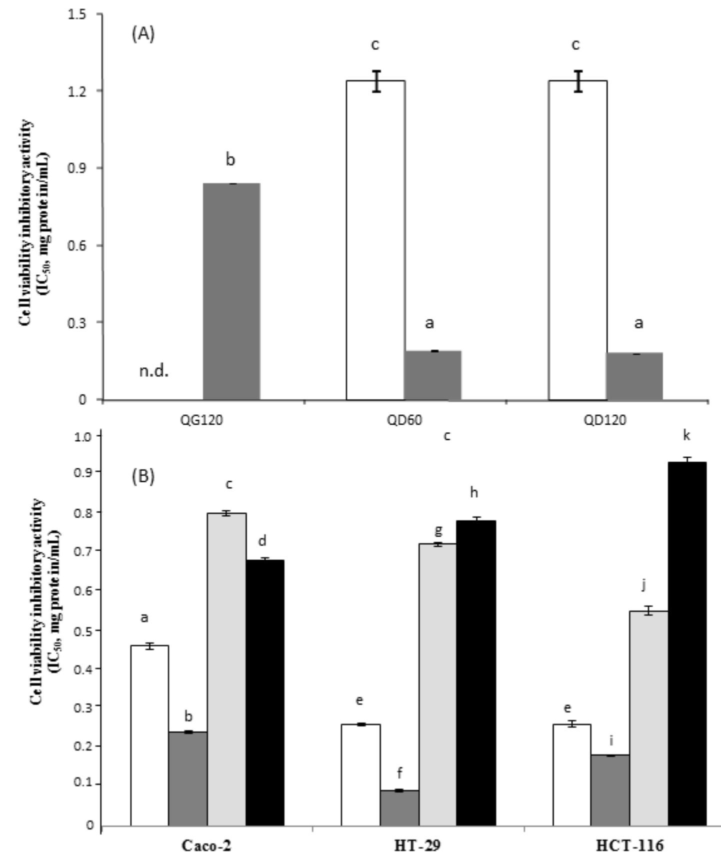

Figure 3

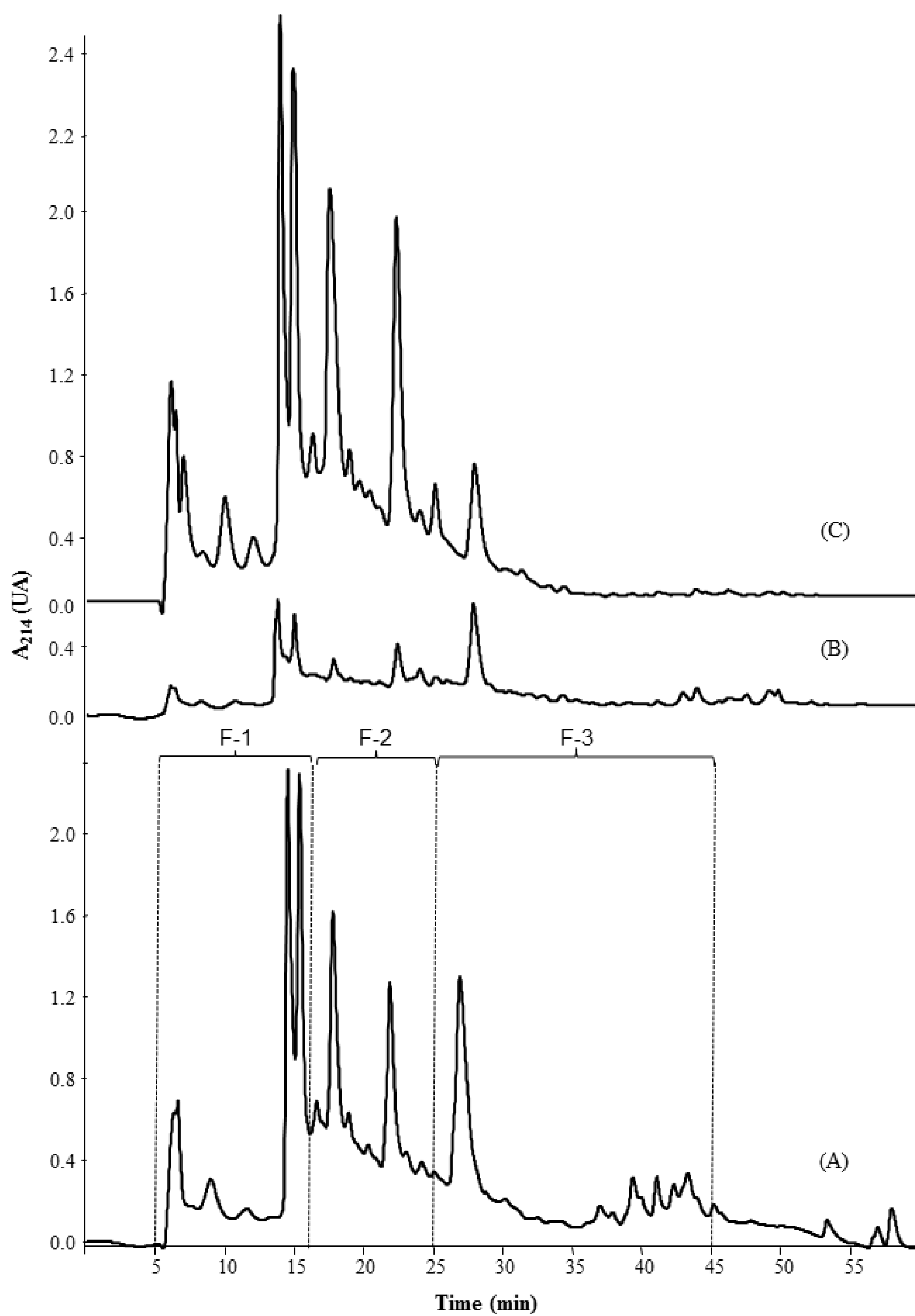

Figure S1

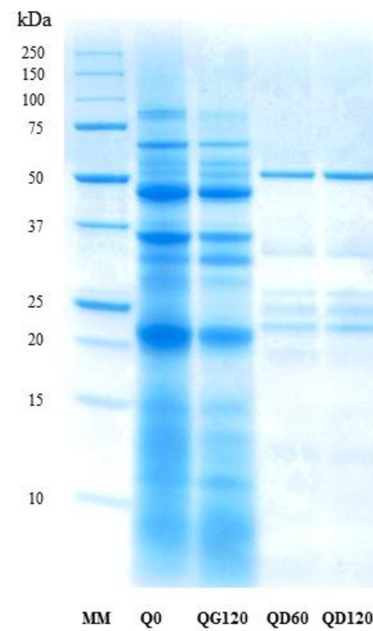

Figure S1. Characterization of the digests obtained after an *in vitro* simulated gastrointestinal digestion by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. MM: molecular weight marker; Q0: non-digested quinoa protein concentrate; QG120: gastric digest after 120 min incubation with pepsin; QD60: after 120 min incubation with pepsin and 60 min incubation with pancreatin; QD120: gastroduodenal digest after 120 min incubation with pepsin and 60 min incubation with pancreatin.

Figure S2

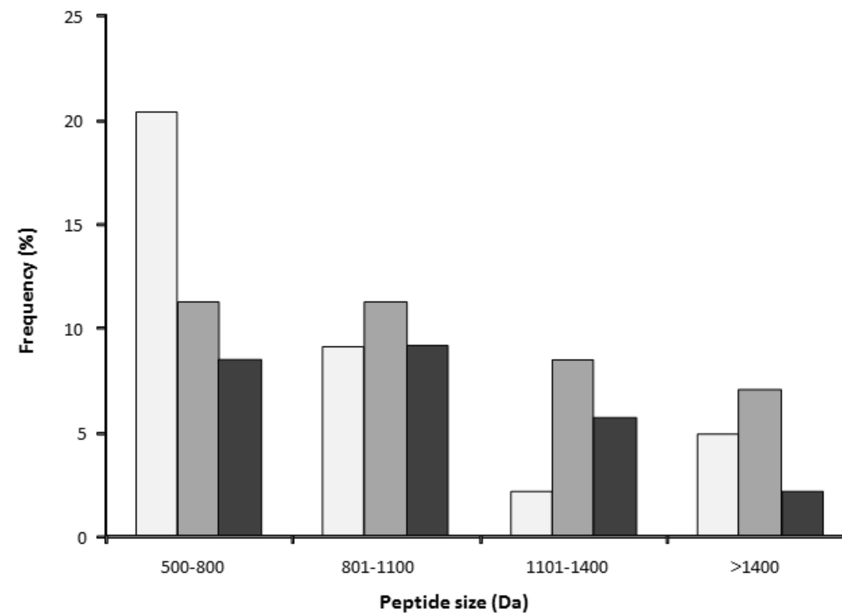

Figure S2. Peptide mass frequency distribution of the fractions (□) F-1, (▒) F-2, and (■) F-3 obtained by preparative RP-HPLC from gastroduodenal digest of quinoa protein concentrate after 120 min incubation with pepsin and 60 min incubation with pancreatin (QD60)

Publicación IV: Digestibilidad de un concentrado proteico de quinua (*Chenopodium quinoa* Willd.) y su potencial para inhibir la peroxidación lipídica en un modelo de larva de pez cebra.

R. Vilcacundo, D. Barrio, C. Carpio, A. García-Ruiz, J. Rúales, B. Hernández-Ledesma, W. Carrillo. 2017. Digestibility of quinoa *(Chenopodium quinoa* Willd.) protein concentrate and its potential to inhibit lipid peroxidation in the zebrafish larvae model. Plant Foods for Human Nutrition, 72, 294-300.

Resumen

Durante el metabolismo celular, el oxígeno es parcialmente reducido dando lugar a moléculas denominadas especies reactivas de oxígeno (ROS). Un exceso de estas especies puede provocar cambios celulares, oxidación de los lípidos de membrana, apoptosis y daños en las enzimas y el DNA que provocan el conocido estado de estrés oxidativo que está relacionado con múltiples desórdenes crónicos. Además, los radicales libres son responsables de las alteraciones en los alimentos y la pérdida de calidad y propiedades organolépticas. Aunque en la actualidad se emplean antioxidantes sintéticos para evitar el deterioro en los alimentos durante el procesado y el almacenamiento, su uso está siendo limitado debido a sus múltiples efectos adversos. Por lo tanto, en los últimos años se buscan nuevos compuestos de origen natural con capacidad antioxidante sin ejercer efectos indeseables. En el presente estudio, se toma de partida el concentrado de proteína de quinua que es sometido a un proceso *in vitro* de digestión simulando condiciones fisiológicas, observándose hidrólisis parcial de las proteínas durante la fase gástrica y su degradación completa tras la incubación con la pancreatina. El digerido gástrico obtenido a pH 1,2 mostró el mayor efecto inhibidor de la peroxidación lipídica (75,15%) empleando el modelo de larva de pez cebra. Este efecto se incrementó hasta el 82,10%, inhibición comparable a la obtenida con el antioxidante sintético butilhidroxitoluol (BHT) usado como control positivo (87,13%).

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ORIGINAL PAPER

Digestibility of Quinoa (*Chenopodium quinoa* Willd.) Protein Concentrate and Its Potential to Inhibit Lipid Peroxidation in the Zebrafish Larvae Model

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Abstract Quinoa protein concentrate (QPC) was extracted and digested under *in vitro* gastrointestinal conditions. The protein content of QPC was in the range between 52.40 and 65.01% depending on the assay used. Quinoa proteins were almost completely hydrolyzed by pepsin at pH of 1.2, 2.0, and 3.2. At high pH, only partial hydrolysis was observed. During the duodenal phase, no intact proteins were visible, indicating their susceptibility to the *in vitro* simulated digestive conditions. Zebrafish larvae model was used to evaluate the *in vivo* ability of gastrointestinal digests to inhibit lipid peroxidation. Gastric digestion at pH 1.2 showed the highest lipid peroxidation inhibition percentage (75.15%). The lipid peroxidation activity increased after the duodenal phase. The digest obtained at the end of the digestive process showed an inhibition percentage of 82.10%, comparable to that showed when using BHT as positive control (87.13%).

Keywords Quinoa protein · Simulated gastrointestinal digestion · Peptides · Inhibition of lipid peroxidation · Zebrafish larvae model

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Introduction

Quinoa (*Chenopodium quinoa* Willd.) is a native pseudocereal of the Andean region in South America, belonging to the *Chenopodiaceae* family [1]. Because of its stress-tolerant characteristics, its gluten-free nature, and its nutritional and biological properties, quinoa has been described as 'one of the grains of the 21st century [2–4].

Oxygen may be partially reduced during normal metabolism to yield reactive molecules termed reactive oxygen species (ROS). An excess of these species can cause cellular changes, oxidation of membrane lipids, apoptosis, and damage to enzymes and DNA. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are used as food additives. However, because of the possible toxic and carcinogenic effects associated to these two antioxidants, their use is legally restricted [5]. Nowadays, there is great interest in finding new natural compounds as an alternative to the use of synthetic antioxidants. In the last years, food protein-derived hydrolysates and peptides with antioxidant activity have attracted the interest [6]. As it has been reported for other activities, *in vitro* simulated gastrointestinal digestion has an important effect increasing the antioxidant activity of food proteins [7–9].

Zebrafish (*Danio rerio*) is an emergent vertebrate model used to study toxicological events, such as neurochemical alterations promoted by heavy metals, new drugs and chemicals [10]. Richetti et al. [11] used the zebrafish larvae model to evaluate lipid peroxidation produced in presence of heavy metals [11]. Recently, this model has been applied to evaluate the antioxidant activity of food compounds. Carrillo et al. [12] reported the lipid peroxidation inhibitory activity of peptides isolated from hen egg white lysozyme using the TBARS method in the zebrafish larvae model [12]. Lipids are biomolecules with great possibilities to undergo oxidation resulting in

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the release of different secondary molecules, mainly aldehydes, capable to increase oxidative damage. Malondialdehyde is the most studied product of polyunsaturated fatty acid peroxidation. Letelier et al. [13] studied the inhibition of lipid peroxidation of polyphenol thiol components of quinoa seeds using the TBARS method [13]. However, to our knowledge, no data about the quinoa proteins potential to release inhibitory peptides of lipid peroxidation in *in vivo* models can be found. The aim of this study was to obtain QPCs from *Chenopodium quinoa* Willd. and to evaluate their digestibility in *in vitro* conditions simulating gastrointestinal digestion. The cytotoxicity and the inhibition of lipid peroxidation in the zebrafish larvae model of gastroduodenal digests were also studied.

Materials and Methods

Obtention of Quinoa Protein Concentrate (QPC)

Quinoa protein concentrate (QPC) was prepared according to Martínez and Añón [14] with some modifications. The defatted flour was suspended in water (1:10, *w*/*v*) adjusting the pH to 8.0. The suspension was stirred for 1 h and then, centrifuged at 4,500 x *g* for 30 min at 25 °C. The supernatant was adjusted to pH 4.0, and centrifuged for 20 min at 4,500 x *g*. The pellet was collected, the pH adjusted with 0.1 M NaOH, lyophilized and kept at −20 °C. The QPC protein content was determined using Biuret, BCA (Pierce, Rockford, IL, USA), and Dumas methods [15–17].

In Vitro Gastrointestinal Digestion of QPC

QPC (5 mg/mL) was subjected to gastric digestion using simulated gastric fluid (0.35 M NaCl) at different pH: 1.2, 2.0, 3.2, 4.5, and 5.5 [18]. Porcine pepsin (EC 3.4.23.1, 4,500 U/mg protein, Sigma-Aldrich, St. Louis, MO, USA) was added with an enzyme/substrate (E/S) ratio of 2000 U/mg. The digestion was performed at 37 °C for 120 min. Aliquots of gastric digests were withdrawn inactivating the enzyme by heating at 80 °C for 5 min. The pH of digest obtained at the end of gastric phase (pH 3.2) was adjusted to 7.0, and intestinal phase started after addition of 0.125 M bile salt mixture (Sigma-Aldrich), 1 M $CaCl_2$ (7.6 mM final concentration), and pancreatin (100 U/mg) dissolved in 20.3 mM Bis-Tris buffer. Reactions were carried out at 37 °C for 120 min, and stopped by heating at 80 °C for 5 min.

Characterization of QPC Digests by SDS–PAGE Electrophoresis and RP-UHPLC

SDS-PAGE of QPC and its gastric and gastroduodenal digests was carried out using 4 and 12% polyacrylamide gels in a Mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, USA). Polypeptide bands were stained with Coomassie Brilliant Blue G-250 for 12 h. Relative molecular masses of protein were determined by comparing them with molecular weight markers (Bio-Rad). Characterization was also carried out by RP-UHPLC using an Agilent 1200 infinity series UHPLC system (Agilent Technologies, Waldbron, Germany). The wavelength detector was 214 nm. The column used was Zorbax EC C18 (Agilent Poroshell 120, 4.6 × 50 mm × 2.7 μm of particle size). Samples were eluted at 1.0 mL/min with a lineal gradient from 0 to 70% of solvent B (acetonitrile and trifluoroacetic acid, TFA, 0.270% *v*/v) in solvent A (water and TFA, 0.370% *v*/v) [19].

Zebrafish Larvae Collection and Toxicity Test

Adult zebrafish was kept on 16 h light and 8 h dark cycles. Embryos were obtained by photo-induced spawning over green plants and then, cultured at 28 °C in a fishbowl. Early larvae were maintained according to Nagel [20]. Post fertilization early larvae zebrafish were maintained according to Kimmel et al. [21]. Five days post fecundation larvae (30 larvae per well) were incubated in 24-well plates. QPC digests were added at three different concentrations (5.0, 2.5, and 1.25 mg/mL) in 200 μL of water. The effect of digests on larvae was measured after 4, 24 and 48 h. At the end of incubation time, larvae mortality and morphologic changes were observed, determining the percentage of dead larvae. Stereoscopic microscope images were taken to obtain a registration of the morphological effects of digests on larvae anatomy and compared to controls.

Thiobarbituric Acid Reactive Substances (TBARS) in Zebrafish Larvae Model

TBARS method was used as described by Carrillo et al. [12]. Larvae were incubated in 24-well plates (30 larvae/well) with 2.0 mg/mL of QPC digests. Groups of 30 larvae/well in aquarium water were used as controls. Lipid peroxidation was initiated by adding 1 mL of 500 μM H_2O_2 and incubated for 8 h at 28 °C. Then, H_2O_2 was removed and 500 μL of Tween 0.1% were added. Larvae were mixed and homogenized with a homogenizer (T25 basic Ultra Turrax IKA, Thermo Fisher Scientific, Germany). 1 mL of 1% TBA was added and subsequently, the solution was heated at 95 °C for 1 h, and then cooled down for 15 min. Absorbance of the final solution containing zebrafish larvae and QPC digests was measured at 532 nm using a spectrophotometer (Thermo Scientific Evolution 200, Germany). The values of antioxidant activity were expressed as the percentage of inhibition of lipid peroxidation in larvae homogenate as follows: % Inhibition of lipid peroxidation = [1-(Ab - As)/Ab × 100], where Ab is the absorbance of blank and As is the absorbance of the sample.

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Statistical Analysis

Results are presented as means ± standard deviation from three replicates of each experiment. Differences between mean values were determined by the analysis of variance (ANOVA). The *post hoc* analysis was performed by the Tukey test. All tests were considered significant at $P < 0.05$ using the software package Prism 4.

Results and Discussion

Characterization of QPC Digested under Simulated Gastrointestinal Digestion

QPC was obtained using an isoelectric precipitation method with water at pH 4.0. The protein content value of QPC, measured by Biuret, Dumas, and BCA methods, were 65.01, 52.4, and 55.50%, respectively. The statistical differences ($p < 0.05$) could be due to the different level of sensitivity of each method. Since the protein extraction method used in our study did not include a lipid extraction phase, residual lipids present in QPC could interfere on the Biuret measurement, increasing the protein value determined by this method. This interference had been previously reported [22]. Therefore, the protein value obtained by Dumas method was used to prepare the samples for further analyses.

QPC and its digests were analysed using SDS-PAGE. As shown in Fig. 1a, the protein profile of QPC showed bands with molecular weights between 6.5 to 200 kDa. The most intense bands were those ranged from 15 to 40 kDa corresponding to 7S and 11S globulins, and 2S albumin from quinoa. These results are in agreement with Abugoch et al. [23] that indicated the presence of quinoa globulins with molecular weight between 20 to 50 kDa, and albumins with molecular weight lower than 20 kDa [23]. Previously, Brinegar et al. 24 identified 7S globulin with molecular weight of 50 kDa, 11S globulin (acid subunit 22–23 kDa and basic subunit 32–39 kDa), and quinoa albumins with molecular weight of 8–9 kDa [24]. In Fig. 1a, quinoa proteins were completely hydrolysed by pepsin when incubation was performed at pH 1.2, 2.0, and 3.2. However, no hydrolysis of quinoa proteins is appreciable at pH 4.5 and pH 5.5. This could be due to the lower enzyme activity shown by pepsin at high pH values. After incubation with pancreatic enzymes, quinoa proteins were totally hydrolysed (Fig. 1b), indicating the high digestibility of these proteins under conditions simulating physiological digestion. No small peptides could be observed because of the composition of the gel (12% acrylamide) used to separate proteins. Gels with 16–20% of acrylamide would allow visualizing bands corresponding to small peptides released during gastrointestinal digestion [25].

Analysis of quinoa protein hydrolysates was also carried out by RP-UHPLC to observe the changes in the molecular weight and hydrophobicity of peptides released during the digestive process. The results obtained are shown in Fig. 2. Four zones, named as F1, F2, F3 and F4 with retention times of 0–1.0, 1.0–4.0, 4.0–5.0 and 5.0–8.0 min, respectively, were clearly identified in the chromatogram for non-digested QPC (Fig. 2a). F1 was constituted by a major peak, which an area corresponding to 36% of the total chromatogram area. F2 showed several small peaks, representing 12% of the total area, and F3 presented two major peaks representing 32% of the total chromatogram area. Finally, two major peaks, which area corresponded to 20% of the total area, were visible in fraction F4. The chromatograms of gastric (at different incubation pH) and gastroduodenal digests are shown in

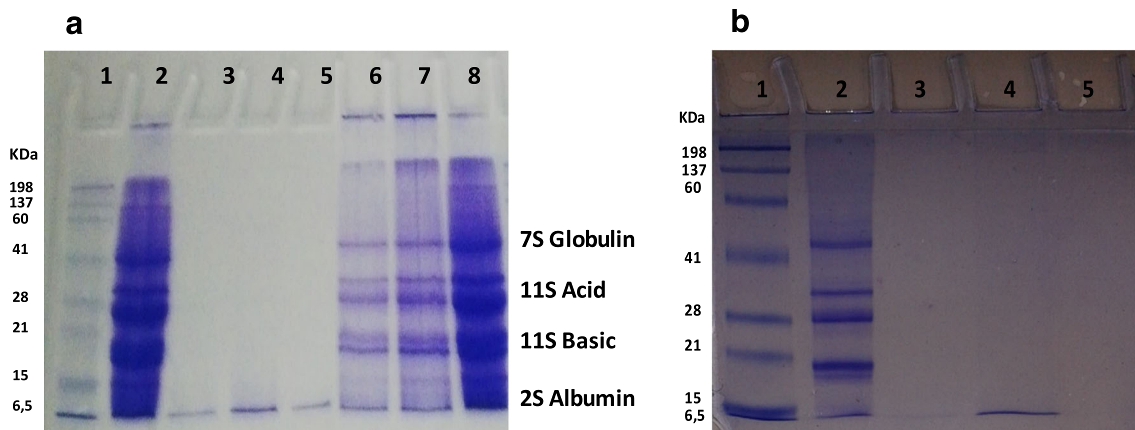

Fig. 1 SDS-PAGE electrophoresis of quinoa protein concentrate (QPC) and its gastric and gastroduodenal digests. **a** Lanes 1: molecular weight marker, lanes 2 and 8: QPC, lane 3: gastric digest obtained at pH 1.2, lane 4: gastric digest obtained at pH 2.0, lane 5: gastric digest obtained at pH 3.2, lane 6: gastric digest obtained at pH 4.5, lane 7: gastric digest obtained at pH 5.5. **b** Lane 1: molecular weight marker, lane 2: QPC, lane 3: gastric digest obtained at pH 3.2, lane 4: gastroduodenal digest, lane 5: water

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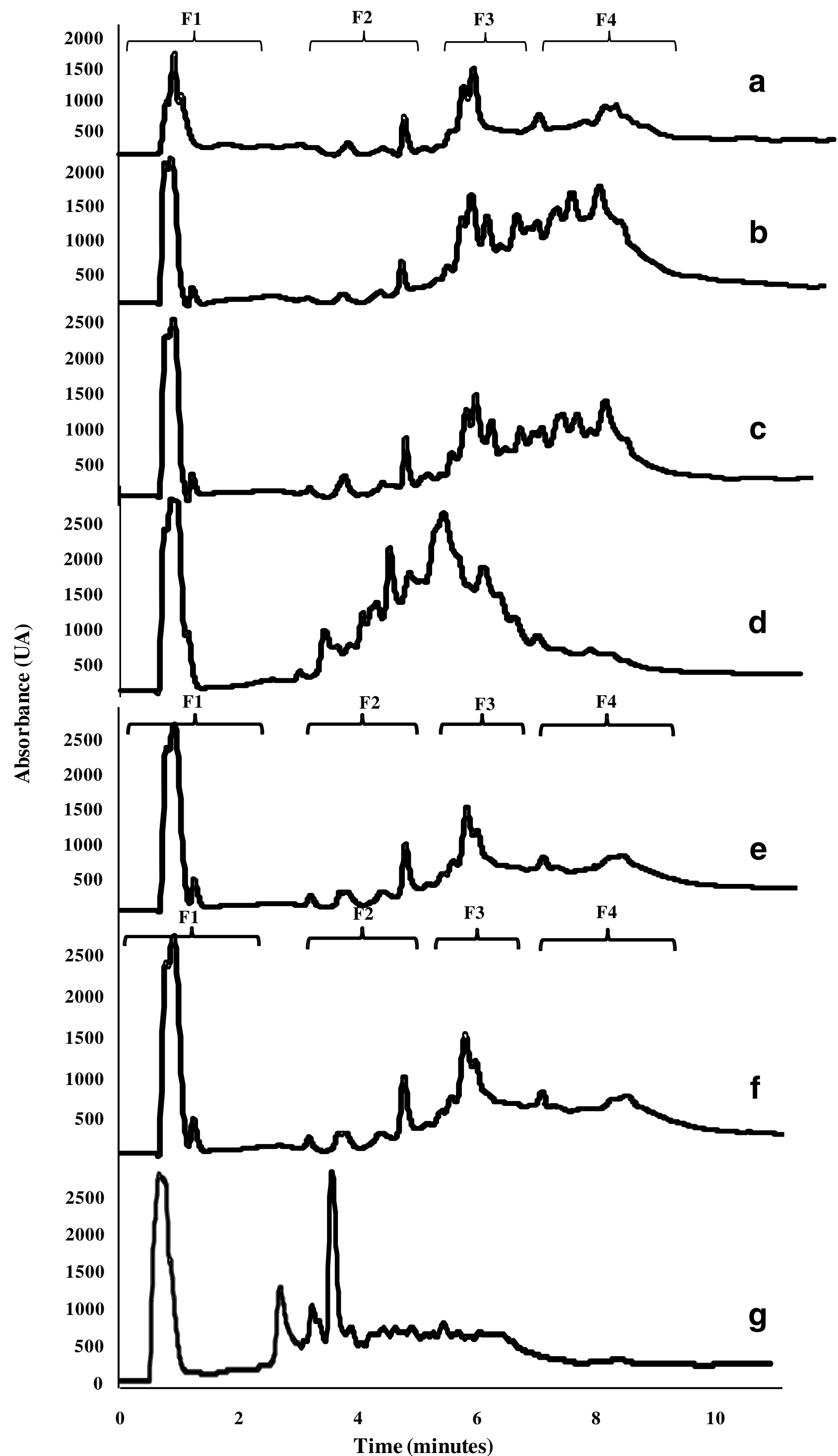

Fig. 2 RP-UHPLC analysis of quinoa protein concentrate (QPC) and its gastric and gastroduodenal digests. (**a**) QPC, (**b**) gastric digest obtained at pH 1.2 (**c**) gastric digest obtained at pH 2.0 (**d**) gastric digest obtained at pH 3.2 (**e**) gastric digest obtained at pH 4.5 (**f**) gastric digest obtained at pH 5.5 (**g**) gastroduodenal digest

Fig. 2b-g. The profiles of gastric digests obtained at pH 4.5 and 5.5 were similar to those obtained for non-digested QPC. This result is in agreement with those found by SDS-PAGE, indicating that no hydrolysis of quinoa proteins by pepsin was appreciable at high incubation pH. However, at lower pH, proteins were hydrolysed by pepsin, and profiles of fractions F2, F3, and F4 notably changed. Mainly, an increase in the intensity of the peaks contained in F3 and F4 was observed in comparison with the intensity of non-digested QPC fractions. The chromatogram obtained at the end of simulated gastrointestinal digestion showed that whereas F1 was conserved, new peaks corresponding to small and hydrophilic peptides appeared after the action of pancreatin (Fig. 2g).

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Antioxidant Activity of Quinoa Protein Digests

The cytotoxicity of QPC digests was evaluated using the *in vivo* zebrafish larvae model. After 48 h of exposure to different concentrations (5.0, 2.5, and 1.25 mg/mL) of digests, the zebrafish larvae exhibited a similar physiology than the larvae control (non-treated) (Fig. 3a). These results indicate that the digests did not show cytotoxic effects. Then, the zebrafish larvae model was used to evaluate the *in vivo* lipid peroxidation inhibitory capacity of QPC digests as it had been previously used for peptides derived from hen egg-white lysozyme [12]. This capacity was determined as the protective effect of hydrolysates against lipid peroxidation induced by hydrogen peroxide in the animal model. The inhibition percentages for the gastric and gastroduodenal digests are shown in Fig. 3b. Among gastric digests, those obtained at lower pH showed higher inhibitory capacity than those obtained at high pH. The inhibition percentage of the digest obtained at pH 1.2 was of 75.15% while the percentage of digest at pH 5.5 was of 16.08%. This fact could be due to the highest hydrolysis of quinoa proteins observed when pepsin acted at its optimum pH, resulting in the release of high number of peptides with a potent ability to inhibit lipid peroxidation. These peptides could be further hydrolysed by pancreatin releasing new fragments with higher antioxidant activity. Thus, the hydrolysate obtained at the end of the digestive process showed the strongest inhibitory capacity with a percentage of inhibition of 82.10%, similar to the positive control (BHT) used in the assay (87.13%).

Previous studies have reported the ability of peptides derived from different food sources to inhibit lipid peroxidation. As an example, Chen et al. [26] reported this ability for fish and shellfish protein hydrolysates [26]. Even though the exact mechanism of peptides to act as antioxidant is not clearly known, the size and the presence of some aromatic amino acids and histidine are reported to play a vital role in this activity [12, 27]. It is expected that hydrophobic peptides can protect linoleic acid easily by donating photons to hydrophobic peroxyl radicals. On the other hand, Mendis et al. [28] have described the ability of hydrolysates derived from jumbo squid (*Dosidicus gigas*) to inhibit linoleic acid peroxidation in an emulsified model system [28]. The fraction lower than 3 kDa obtained by ultrafiltration showed an inhibition percentage of 82.0% indicating that small peptides released during hydrolysis were the main responsible for the observed effects. Sequences Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu and Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg were identified as the major contributors of the antioxidant activity, with inhibition percentages of 66 and 72%, respectively [28]. The presence of amino acids Phe and Leu has been suggested important to exert the antioxidant effects. Quinoa proteins are known to be rich in amino acids Leu, Hys, Phe and Arg, thus peptides containing these amino acids might be released during digestive processes and exert antioxidant activity through inhibiting lipid peroxidation [29]. Leu and Arg were also present in antioxidant sequences reported by Carrillo et al. [12] obtained from hen egg lysozyme hydrolysate by pepsin [12].

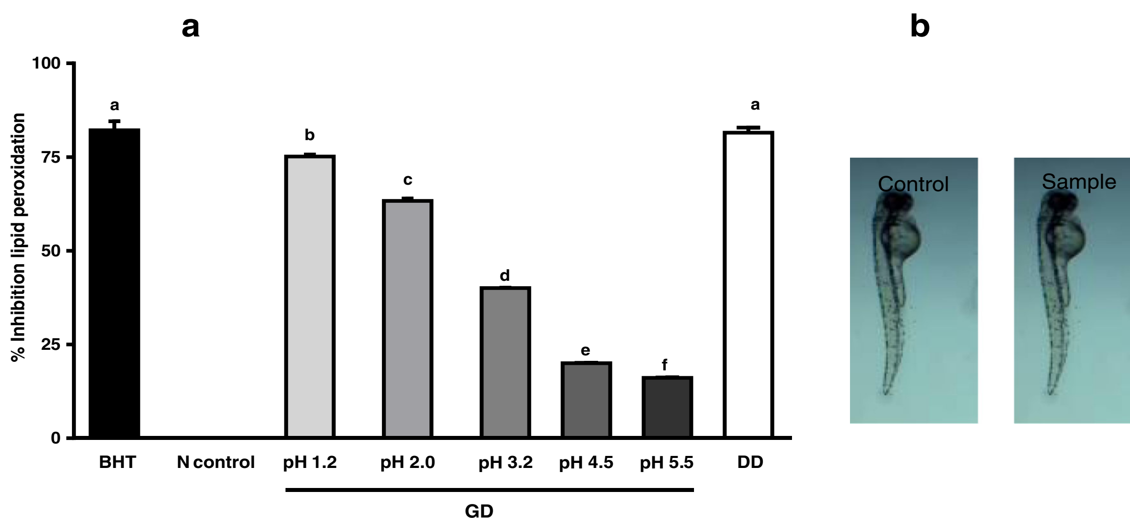

Fig. 3 **a** TBARS activity. BHT: positive control (BHT 0.1 mg/mL); N control: negative control (water); GD: gastric digests obtained at pH 1.2, 2.0, 3.2, 4.5, and 5.5 (concentration of 2.0 mg/mL). DD: gastroduodenal digest (concentration of 2.0 mg/mL). **b** Effect on cell morphology of zebrafish larvae. Zebrafish were incubated for 48 h with 5.0 mg/mL gastroduodenal digest (sample) in comparison with control. Magnification 40× of stereoscopic microscopy

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Conclusion

In summary, results of this study demonstrate, for the first time, that quinoa protein hydrolysates obtained during an *in vitro* simulated gastrointestinal digestion are capable of inhibiting lipid peroxidation in the *in vivo* zebrafish model. Therefore, quinoa is a promising source of antioxidant peptides after its gastrointestinal digestion that could exert a preventive effect against oxidative stress-associated disorders. Moreover, quinoa derived hydrolysates might be used as natural additive and/or preservative in foodstuff to control the lipid oxidation responsible for deterioration of food during production and storage. Further studies would be needed to identify the peptides responsible for the antioxidant activity, and to study in depth the *in vivo* mechanisms of action.

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Compliance with Ethical Standards All animal work conformed to ethical guidlines and was approved by relevant local animal ethics committees.

Conflict of Interest The authors declare that they have no conflict of interest.

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Proteínas de amaranto (*Amaranthus caudatus*): caracterización y potencialidad como fuente de péptidos multifuncionales tras su digestión gastrointestinal *in vitro*

2.5 Publicación V: **Identification of proteins isolate from amaranth (Amaranthus caudatus) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with water and NaCl 0.1 M solvents.**

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH, 9 (2016) 331-334

2.6 Publicación VI: **Release of multifunctional peptides from kiwicha (*Amaranthus caudatus*) protein under *in vitro* simulated gastrointestinal digestion.**

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY (*submitted*)

2.7 Publicación VII: **Evaluation of the lipid peroxidation inhibitory capacity of Kiwicha (*Amaranthus caudatus*) protein digested under simulated gastrointestinal conditions.**

JOURNAL OF MEDICINAL FOOD (submitted)

Publicación V: **Identificación, mediante SDS-PAGE, de las proteínas de un aislado de amaranto (*Amaranthus caudatus*) extraídas empleando agua y NaCl 0,1 M como solventes.**

C. Acosta, C. Carpio, R. Vilcacundo, W. Carrillo. 2016. Identification of proteins isolate from amaranth (*Amaranthus caudatus*) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with water and NaCl 0.1 M solvents. Asian Journal of Pharmaceutical and Clinical Research, 9, 331-334.

Resumen

El amaranto (*Amaranthus caudatus*) es un pseudocereal originario de las regiones andinas de Sudamérica pero cultivado actualmente en todo el mundo. Junto con *Amaranthus hypocondriacus* y *A. cruentus, A. caudatus* es una de las tres especies comestibles de este género. Presenta un excelente valor nutricional debido fundamentalmente a su alto contenido en proteínas, superior al encontrado en la mayoría de los cereales y de alta calidad y digestibilidad. Para llevar a cabo la extracción de aislados proteicos vegetales de gran relevancia actual en la industria alimentaria se emplea un pH ácido de precipitación de las proteínas, en el rango entre 4 y 6 tras ser solubilizadas a un pH alcalino. En este estudio se llevó a cabo la optimización del proceso de extracción de proteínas de amaranto (*A. caudatus*) usando pH alcalino de solubilización y diferentes pHs de precipitación usando agua y NaCl como solventes. La caracterización del perfil proteico se llevó a cabo mediante electroforesis en gel SDS-PAGE. La banda de peso molecular 50 kD correspondió a la globulina 7S mientras que las bandas de peso 36-38 kDa y 18-20 kDa correspondieron a las dos subunidades, ácida y básica de la globulina 11S. Las bandas de peso molecular inferior a 14,4 kDa correspondieron a las albúminas.

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Research Article

IDENTIFICATION OF PROTEINS ISOLATE FROM AMARANTH (*AMARANTHUS CAUDATUS*) BY SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS WITH WATER AND NACL 0.1 M SOLVENTS

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ABSTRACT

Objective: The aim of this study was to obtain protein isolate from amaranth using alkaline method at extraction pH 8 and extraction pH 12 with different precipitation pHs and to analyze protein isolate with electrophoresis.

Methods: Amaranth protein isolates were obtained using isoelectric precipitation method at different pHs. Proteins were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Results: A yield of 20.52% of protein isolate of defatted amaranth at pH 4.0 was obtained. The content of protein isolate was higher than 53% in all pH assays. Globulins and albumins in protein isolate at different pHs were observed. A band with 50 kDa corresponding to 7S globulin was found. The bands 36-38 kDa and 18-20 kDa correspond to 11S globulin. Bands less to 14.4 kDa correspond to albumins.

Conclusions: Amaranth protein isolate is possible to obtain in extreme conditions of pH. The treatment with water was optimum to obtain amaranth protein isolate.

Keywords: Amaranth, Globulins, Albumins, Proteinisolate, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

INTRODUCTION

Amaranth (*Amaranthus caudatus*) is a pseudocereal cultivated around the world in the Andean regions of South America, Asian, Africa and Europe and belongs to the family Amaranthaceae. Amaranth has more of 250 species but only three species are comestible as grain: *A. caudatus, Amaranthus hypocondriacus,* and *Amaranthus cruentus.* The protein content of amaranth is higher (13-19%) than most cereals [1-3]. This is an option as a source of proteins, producing bioactive peptides that prevent chronic diseases. The use of protein isolate has increased in the food industry because of different factors such as higher protein levels, good functionality, bioactive components, and lower content of anti-nutritional factors [4]. The most used method to obtain protein isolate is alkaline pH (8-11) through solubilization of proteins at acid pH (4-6) for their isoelectric precipitation [5]. The aim of this study was to obtain protein isolate from amaranth using alkaline pH at different pHs of precipitation using water and NaCl as solvents and to analyze these proteins with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

METHODS

Amaranth flour and proximate analysis

Amaranth flour was defatted through extraction with hexane (1:10 w/v) at room temperature during 24 hrs, under continuous stirring during the first 5 hrs. After drying at room temperature, the flour was stored at 4°C until used. Analytical methods such as moisture, fat, total fiber, and soluble solids contents were determined according to the methods of AOAC [6], numbers 9250.10, 930.09, 985.29, and 923.03, respectively. The protein content of the sample was determined by the micro-Kjeldahl method AOAC number 920.152, % (N×6.25). Carbohydrates percentage was calculated with the formulas: % Carbohydrates=100−(% moisture+% proteins+% fat+% soluble solids+% total fiber). Contents were expressed on a dry weight basis.

Protein isolate from amaranth

Amaranth isolate was prepared according to Martıínez and Añón [7] with modifications. The defatted flour was suspended in water and 0.1 M NaCl in a 1:10 w/v, and the suspension was adjusted to pH 8.0 and 12.0 by adding 1 M NaOH. The suspension was stirred during 1 hr and then centrifuged at 4500 g for 30 minutes at 25°C. The supernatant was adjusted to pHs 2.0, 3.0, 4.0, 5.0, and 6.0 with 2 N HCl and centrifuged for 20 minutes at 4500 g. The pellet was suspended in a small volume of water, neutralized with 0.1 M NaOH, and lyophilized and then frozen at −20°C. The content of protein isolate was determined using the method biuret [8]. The amaranth protein isolate obtained with salt were dialyzed in Milli-Q water with a membrane of 5000 Da of pore size (spectra/por) during 24 hrs at room temperature. The samples were subsequently lyophilized and then frozen at −20°C.

SDS-PAGE

SDS-PAGE of amaranth protein isolate was carried out according to the method proposed by Laemmli [9] using 4-12% polyacrylamide gel in a Mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, USA). Polypeptide bands were stained in Coomassie Brilliant Blue R-250 for 12 hrs. Relative molecular masses of protein were determined by a comparison to molecular weight (MW) markers (Bio-Rad, Hercules, CA, USA) and software Quantity One of Chemidoc (Bio-Rad).

Statistical analysis

Results are presented as means±standard deviation (SD) from three replicates of each experiment. Differences between mean values were determined by the analysis of variance (ANOVA). The *post hoc* analysis was performed by the Tukey's test. All tests were considered significant at $p<0.05$. A statistical analysis was performed using the software package Prism 4 for Windows, version 4.3 (GraphPad Software Inc., www.graphpad.com).

RESULTS

Composition analysis

Table 1 shows the approximate composition of defatted amaranth flour obtained with water. The protein content was 13.9%; this result is in accordance with other authors [10,11]. Moreover, we can see that the approximate composition of amaranth protein isolate at pH 4.0 shows the protein content increase to 50.8% using Kjeldahl method for determination of protein content (%N×6.25). Carbohydrates content in this protein isolate resulted to be low at 30.47%. There is then a statistical difference (p<0.05) between defatted flour and protein isolate as shown in Table 1. The content of protein also was determined with biuret method; the content of protein increases from 13.9% in the amaranth flour to 82.33% in the proteins isolate at pH 4.0 with water solvent. All pHs analyzed resulted in a content of proteins higher than 50% (Table 2).

Results represent the average of three determinations±SD. Different letters show a statistical difference between the groups (columns) (<0.05) ANOVA and Turkey's test.

Table 1: Proximate analysis of DAF and AIP

%	Protein	Fat	Moisture	Total fibre	Soluble solids	Carbohyidrates
DAF	13.9[a]	6.64[a]	9.58[a]	0.70[a]	2.74[a]	66.4[a]
AIP	50.82[b]	3.57[a]	6.75[a]	0.51[a]	8.10[b]	30.47[b]

DAF: Defatted amaranth flour, AIP: Amaranth isolate protein, SD: Standard deviation, results represent the average of three determinations ±SD. Different letters show statistical difference from the control group (*p* <0.05) ANOVA and Turkey's test

Table 2: Protein content in amaranth isolate obtained at extraction pH 8 and extraction pH 12 with different precipitation pHs

Treatment	Isolate at pH 8% of protein content (%)		Isolate at pH 12% of protein content (%)	
	Agua	NaCl	Agua	NaCl
pH 2.0	73.83±3.91[a]	1.01±0.60[a]	53.39±2.38[a]	16.41±0.92[a]
pH 3.0	59.30±2.18[b]	2.22±0.79[a]	83.42±1.39[b]	16.88±0.77[a]
pH 4.0	82.33±2.37[c]	23.91±1.01[b]	73.10±3.37[c]	19.30±3.51[a]
pH 5.0	56.70±2.60[b]	37.66±4.61[c]	53.01±2.40[a]	17.75±1.35[a]
pH 6.0	58.98±2.18[b]	0.02±0.00[a]	73.61±2.11[c]	30.32±3.09[b]

SD: Standard deviation, results represent the average of three determinations ±SD. Different letters show statistical difference from the control group (*p* <0.05) ANOVA and Turkey's test

Results represent the average of three determinations±SD. Different letters show a statistical difference between the groups (columns) (<0.05) ANOVA and Turkey's test.

Effect of pH on the extraction of amaranth proteins

Extraction at pH 8 at different pHs

The protein yields of amaranth protein isolate solubilized at pH 8 precipitate at different pHs (2.0, 3.0, 4.0, 5.0 and 6.0) were obtained using water and NaCl 0.1 M as solvents.

Using water as solvent, the highest yield was obtained at pH 4.0 with a 20.52% of protein isolate content, whereas using NaCl 0.1 M as solvent, the highest yield at pH 4.0 was 53.77%. NaCl was apparently effective for solubilizing protein from amaranth flour. However, samples using NaCl as solvent were dialyzed with a membrane with porous of 5,000 Da to eliminate the content of salt. Yield after dialysis has statistical differences with respect to using only water as solvent. Yield results of dialyzed NaCl solution were low in all pHs assay with values of 8.46% to pH 4.0 (Table 3).

Values are expressed in grams per 100 g of protein. Values are means±SD of three determinations. Different letters show statistical difference between the groups (file) (<0.05) ANOVA and Turkey's test.

Extraction at pH 12 at different pHs

The protein yields from amaranth protein isolate solubilized at pH 12 precipitate at different pHs (2.0, 3.0, 4.0, 5.0, and 6.0) were obtained using the isoelectric precipitation method with water and NaCl 0.1 M. At extraction pH 12 using water as solvent at pH 4.0, the protein yield obtained was 19.31% while the protein yield obtained with NaCl 0.1 M was 44.57%. After dialysis, the protein yield was 9.33%. At precipitation pH 6.0, the protein isolate presents the highest yield with a value of 17.46% compared to the extraction pH 8.0 with precipitation pH 6.0 which presents a value of 8.31% (Table 4). The extraction of amaranth protein isolate at pH 12 using NaCl as solvent presents a higher protein content than extraction pH 8.0 using NaCl (Table 2).

Values are expressed in grams per 100 g of protein. Values are means±SD of three determinations. Different letters show statistical difference between the groups (file) (<0.05) ANOVA and Turkey's test.

SDS-PAGE

Amaranth seed shows proteins fractions of globulins and albumins as storage protein. Globulins, albumins, and glutelins are the major proteins fractions in amaranth seeds. Its globulin is composed primarily of the 11S globulin. 7S globulin is also present but only in minor quantity [12]. Globulins have two groups depending on its sedimentation coefficient:

Table 3: Content of amaranth protein isolate obtained at extraction pH 8.0 with different precipitation pHs

% Yield	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0
Isolate/water	18.66±0.39[a]	18.66±0.26[a]	20.52±0.38[a]	15.02±0.20[b]	8.31±0.39[c]
Isolate/NaCl (0.1 M)	40.53±0.39[a]	53.62±1.45[b]	53.77±0.15[b]	50.61±0.63[b]	48.32±0.29[c]
Isolate after dialysis	6.64±0.15[a]	8.41±0.11[a]	8.46±0.40[a]	7.21±0.73[a]	6.67±0.59[a]

SD: Standard deviation, results represent the average of three determinations ±SD. Different letters show statistical difference from the control group (*p* <0.05) ANOVA and Turkey's test

Table 4: Content of amaranth protein isolate obtained at extraction pH 12.0 with differentprecipitation pHs

% Yield	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0
Isolate/water	14.78±0.35[a]	17.11±0.46[a]	19.31±0.77[b]	19.11±0.46[b]	17.46±0.62[a]
Isolate/NaCl (0.1 M)	48.89±0.50[a]	39.28±0.46[a]	44.57±0.32[a]	41.06±0.61[b]	53.01±0.58[c]
Isolate after dialysis	8.33±0.14[a]	5.25±0.25[a]	9.33±0.28[a]	5.50±0.25[a]	8.41±0.38[a]

SD: Standard deviation, results represent the average of three determinations ±SD. Different letters show statistical difference from the control group (*p* <0.05) ANOVA and Turkey's test

11-12S and 7-8S. Amaranth storage protein predominant is globulins 7S and 11S. 7S present MW of 41-45 kDa. Recently, globulin 11S from amaranth has been named amaranthine [13]. This protein has two subunits consisting of an acid polypeptide (AS) (33-36 kDa) and a basic polypeptide (AB) (16-19 kDa). The 2S albumins have been described like a band of low MW near 6-10 kDa.

SDS-PAGE at reduced and nonreduced conditions of amaranth-extracted protein in water and salt at extraction pH 8 and 12 with different pHs were assayed. Fig. 1 shows SDS-PAGE of amaranth protein isolate obtained at pH 8 with different precipitation pHs. Proteins mass was determined with software Quantity one of Chemidoc™ PM (Bio-Rad). In the presence of 2-β-mercaptoethanol, proteins with MW ranging between 6.5 and 50 kDa were found in all pHs. The protein bands with 50 kDa corresponding to 7S globulin were found in all pHs assayed. Proteins with MW 36-38 kDa corresponding to 11S AS were found in all pHs with high expressions. On the other hand, proteins with MW 18-20 kDa corresponding to 11S AB were found in all pH values. Proteins with 20-36 kDa correspond to Amaranthine. All proteins bands <15.4 kDa corresponding to albumin components were found in high expressions in all pHs.

SDS-PAGE without 2-β-mercaptoethanol present a similar profile of proteins at all pHs assay, the band with MW 6.5, 15.4, 36, 38 and 50 kDa was found in all pHs with high expression (Fig. 2). Only the bands corresponding at 11S basic (18-20 kDa) not found in the gel at all pHs.

SDS-PAGE with 2-β-mercaptoethanol present complex profile of proteins isolate at pH 12 in all pHs assay. Bands ranging between 6.5 and 50 kDa were observed. This profile has many bands when compared to isolate obtained at pH 8 of solubilization. 7S, 11S globulins and 2S albumins were observed in all pHs assay (Fig. 3).

SDS-PAGE of amaranth isolate solubilized at pH 8 obtained with NaCl 0.1 M was analyzed. Fig. 4 shows profiles at different pHs of precipitation. Bands with MW of 30 kDa were found in all pHs with high expression. At pH 4 and pH 5 bands range between 6.5 and 50 kDa were observed with high expression. When compared with the results of biuret method, we observed a correlation because pH 4 and 5 has high protein content.

DISCUSSION

It is known that two of the major types of storage proteins in legume and some no legume seeds are 7S and 11S based on their sedimentation coefficients. Amaranth seeds, due to their high protein content, are actually the subject of many investigations as potential food source and functional food [14,15]. Different studies have reported high isolate protein yield obtained with NaCl. Achouri *et al.* [16,17] have reported that sesame protein isolates obtained with water (12.5%) and salt (54.6%). We can observe that this yield is apparently higher in salt than water. However, after dialysis this difference was clarified as the increase of weight is due to the weight of the salt.

CONCLUSIONS

The content of proteins was higher than 53% in all pHs assays. Albumins and globulins were identified in amaranth protein isolates

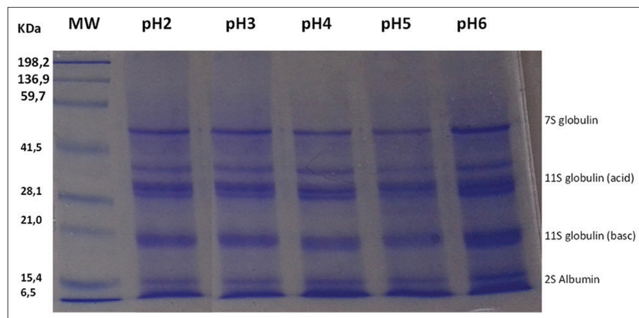

Fig. 1: Electrophoresis sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of amaranth proteins obtained at pH 8 with different precipitation pHs extracted under reducing conditions (sodium dodecyl sulfate+2-β-mercaptoethanol). MW: Molecular weight marker

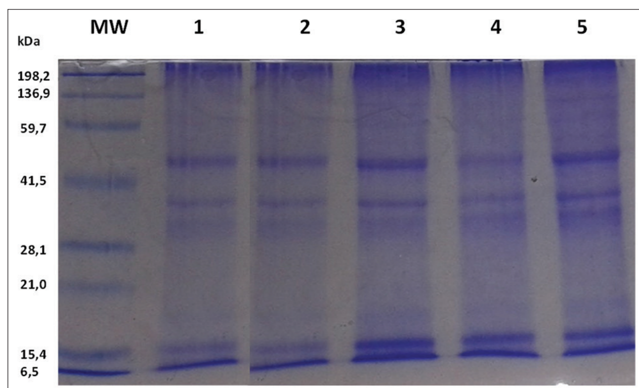

Fig. 2: Electrophoresis sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of amaranth proteins obtained at pH 8 with different precipitation pHs extracted without reducing conditions. MW: Molecular weight marker

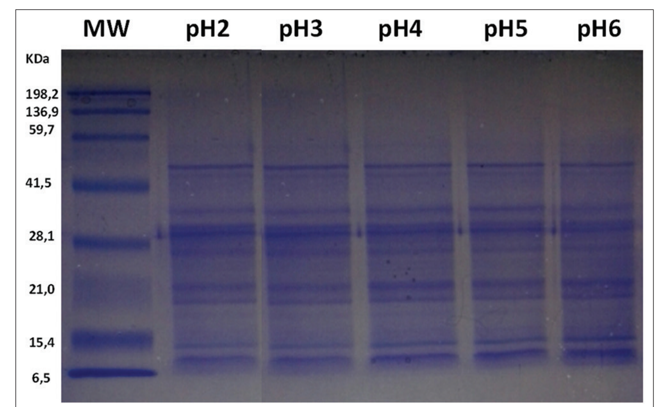

Fig. 3: Electrophoresis sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of amaranth proteins obtained at pH 12 with different precipitation pHs extracted under reducing condition (sodium dodecyl sulfate+2-β-mercaptoethanol). MW: Molecular weight marker

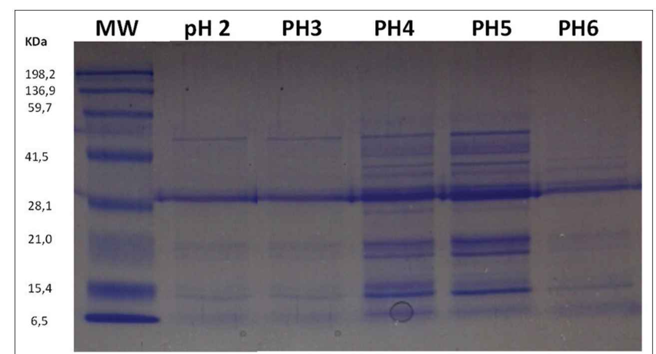

Fig. 4: Electrophoresis sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of amaranth proteins obtained at pH 8 with different precipitation pHs using NaCl 0.1 M as solvent, extracted under reducing condition (sodium dodecyl sulfate+2-β-mercaptoethanol). MW: Molecular weight marker

using isoelectric precipitation at different pHs. It is possible to obtain amaranth protein isolate in extreme conditions with extraction pH 12. The isolate yield is higher using water as solvent rather than using salt as solvent because after dialysis the isolate protein yield is low. Amaranth is a good candidate for supplementation of food protein or substitution of common cereal grains and can be a source of bioactive components.

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Publicación VI: Liberación de péptidos multifuncionales a partir de la proteína de kiwicha (*Amaranthus caudatus*) bajo un proceso de digestión gastrointestinal *in vitro* simulada.

R. Vilcacundo, C. Martínez-Villaluenga, B. Miralles, B. Hernández-Ledesma. Release of multifunctional peptides from kiwicha (*Amaranthus caudatus*) protein under *in vitro* simulated gastrointestinal digestion. Journal of Agricultural and Food Chemistry. Enviado para su publicación.

Resumen

El origen multifactorial de muchos desórdenes crónicos ofrece un nuevo marco de actuación para el desarrollo de nuevos alimentos multifuncionales. En este estudio, se ha investigado el efecto de la digestión gastrointestinal *in vitro* simulando condiciones fisiológicas sobre la liberación de péptidos multifuncionales a partir de la proteína de kiwicha (*Amaranthus caudatus*). El digerido obtenido al final de la fase gástrica presentó la mayor actividad inhibidora de la enzima convertidora de angiotensina (ECA) implicada en el control de la tensión arterial. El digerido duodenal obtenido tras 60 minutos de incubación con la pancreatina mostró la mayor actividad antioxidante, inhibidora de las enzimas dipeptidil peptidasa IV (DDP-IV) y α-amilasa e inhibidora de la viabilidad de células de cáncer de colon Caco-2. Los péptidos de pequeño tamaño (< 5 kDa) fueron los principales responsables de la actividad antioxidante e inhibidora de las diferentes enzimas, mientras que la fracción que contenía los péptidos de mayor tamaño (> 5 kDa) fue más efectiva inhibiendo la viabilidad de las células cancerosas. Se identificaron 13 péptidos derivados de las proteínas de amaranto. El estudio de la relación entre estructura y actividad estableció que entre estos fragmentos, las secuencias FLISCLL, SVFDEELS, and DFIILE presentan alto potencial como multifuncionales por su actividad antioxidante, inhibidora de la ACE y de la α-amilasa. La presencia de estos péptidos podría explicar la multifuncionalidad de los digeridos de proteína de kiwicha.

Release of multifunctional peptides from kiwicha (*Amaranthus caudatus*) protein under *in vitro* simulated gastrointestinal digestion

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Abstract

The multifactorial origin of many chronic diseases provides a new framework for the development of multifunctional foods. In this study, the effect of *in vitro* simulated gastrointestinal digestion of kiwicha (*Amaranthus caudatus*) proteins on the release of multifunctional peptides was evaluated. Gastric digest showed higher angiotensin-I converting enzyme (ACE) inhibitory activity while 60 minutes gastroduodenal digest showed the highest antioxidant, dipeptidyl peptidase IV (DPP-IV), α-amylase, and Caco-2 cell viability inhibitory activities. Peptides < 5 kDa were mainly responsible for the antioxidant, ACE, DPP-IV, and α-amylase inhibitory activities whereas peptides > 5 kDa were more effective inhibiting colon cancer cell viability. Thirteen peptides from amaranth sequenced proteins were identified. The structure-activity relationship analysis of the identified sequences directed to three amaranth fragments, FLISCLL, SVFDEELS, and DFIILE, as potential peptides able to concurrently exert antioxidant capacity and ability to inhibit both ACE and α-amylase. Their presence might explain the multifunctional *in vitro* activity exerted by kiwicha protein digests.

Keywords: kiwicha; simulated gastrointestinal digestion; bioactive peptides; multifunctional activity

1. Introduction

Non-communicable or chronic diseases, particularly cardiovascular and neurodegenerative disorders, respiratory diseases, cancer, and diabetes, are the leading cause of death and disability globally.[1] The majority of non-communicable diseases are generated by four risk factors, with the diet making the biggest contribution, larger than tobacco, alcohol and physical inactivity combined. Generally, certain more highly processed foods such as processed meats and sugar-sweetened beverages have harmful effects whereas minimally processed, bioactive-rich foods like fruits, vegetables, nuts/seeds, beans/legumes, and whole grains have protective effects.[2] A healthy diet combined with the intake of functional foods may help minimizing or even preventing certain chronic diseases.[3] Thus, in the last years, the research interest in bioactive food compounds as an alternative to pharmacological treatment has increased.[4]

In addition to their nutritional quality for providing energy and essential amino acids, food proteins are recognized by their role as source of bioactive peptides. These peptides are encrypted in the protein sequences, but once they are released by gastrointestinal digestion and/or food processing, they can exert a wide range of biological functions such as antihypertensive, antioxidant, anti-inflammatory, anti-diabetic, opioid, immunostimulating, and anti-cancer, among others. This has given rise to intensive research into the potential applications of food-derived multifunctional peptides in the prevention and/or treatment of non-communicable diseases.[5,6]

Kiwicha (*Amaranthus caudatus*) is an ancestral pseudocereal commonly produced in Mexico, Peru and other Andean countries. Similarly to other *Amaranth* species, its production is rapidly growing around the world due to its agronomical and nutritional value, and its gluten-free nature. It is a fast growing crop that can be cultivated throughout the year under adverse environmental conditions (dryness, high

temperatures, and saline soils) that are inhospitable to conventional cereal crops.[7] Moreover, it has a great nutritional potential as source of lipids (5-13%), specially unsaturated fatty acids, fiber (11-23%), minerals, vitamins, and proteins, whose concentration (13%-19%) is higher than that present in cereals.[8] Amaranth proteins show high digestibility and an excellent balance of essential amino acids such as lysine and sulfur-containing amino acids, which are commonly deficient in traditional cereals and legumes, respectively.[9] Besides its nutritional characteristics, studies have reported the presence of bioactive compounds such as flavonoids, phenolic acids, anthocyanins, tannins, and phytosterols in amaranth grains.[10,11] Thus, the introduction in the diet of amaranth seeds has been associated with health promotion and prevention of chronic diseases such as cancer, cardiovascular disease, diabetes, and hypercholesterolemia.[12] Among the bioactive compounds present in amaranth, peptides have gained interest in the last years. The studies have mainly focused on proteins from *A. hypochondriacus* as source of angiotensin I-converting enzyme (ACE) inhibitory and anti-inflammatory peptides.[13-16] However, studies focused on less broadly exploited amaranth species such as *A. caudatus* (kiwicha) or *A. cruentus* are very scarce.[17,18] although their use is increasingly promoted in countries such as Ecuador or Peru. The objective of this work was to bring in a wide approach by evaluating the antioxidant, ACE, dipeptidyl peptidase IV (DPP-IV), α-amylase, and colon cancer cell viability inhibitory activities of kiwicha protein concentrate *in vitro* hydrolyzed under conditions simulating gastrointestinal digestion. Peptide identification and structure-activity analysis was performed with the aim to ascribe the observed biological activities to particular sequences in a realistic way.

2. Materials and Methods

2.1. Materials

Pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas (EC 232-468-9), porcine bile extract, Pefabloc® SC, ACE (peptidyl-dipeptidase A, EC 3.4.15.1), fluorescein disodium (FL), 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), the tripeptide IPI called diprotin A, human recombinant DPP-IV enzyme, potato soluble starch, and porcine pancreatic α-amylase (EC 3.2.1.1) were purchased from Sigma-Aldrich (Madrid, Spain). Fluorogenic substrate *o*-aminobenzoylglycyl-*p*-nitro-l-phenylalanyl-l-proline (Abz-Gly-p-Phe(NO_2)-Pro-OH) was from Bachem Feinchemikalien (Bubendorf, Switzerland), and chromogenic substrate (H-Gly-Pro-*p*-nitroaniline) from Enzo Life Sciences Inc. (Farmingdale, NY, USA). 2,2'-azobis (2-methylpropionamide)-dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Aldrich (Milwaukee, WI, USA). The rest of chemicals used were of HPLC grade.

2.2. Preparation of kiwicha protein concentrate

The kiwicha protein concentrate (KPC) was prepared following the protocol of Acosta and coworkers,[19] with slight modifications. Kiwicha flour (Gramolino, Quito, Ecuador) was suspended in water (1:10, w/v) and its pH was adjusted to 12.0 with 1 M NaOH. The suspension was stirred for 1 h and centrifuged at 4,500 x *g* for 30 min at 25ºC. After adjusting the pH of the supernatant to 4.0 with 2 N HCl, it was centrifuged at 4,500 x *g* during 20 min at 4ºC. The pellet was dissolved in a small volume of water, neutralized with 0.1 M NaOH, lyophilized, and kept at -20ºC until further analyses. The protein content of KPC was determined by Kjeldahl.

2.3. In vitro simulation of gastrointestinal digestion of kiwicha protein concentrate

KPC was digested following the *in vitro* harmonized protocol of Minekus et al.[20] Briefly, KPC was dissolved in water (700 mg/5 mL) and then, diluted with simulated gastric fluid containing pepsin (2000 U/mL in the final mixture, ratio of 50:50, v/v). The mixture was incubated at 37°C in an orbital shaker at 150 rpm, withdrawing aliquots at the starting point (K0) and after 120 min of gastric digestion (KG120). The enzymatic reaction was stopped by adjusting the pH at 7.0 with 1 M NaOH and snap freezing in liquid nitrogen. Gastric phase was mixed with simulated intestinal fluid containing pancreatin (100 U trypsin activity/mL of final mixture, ratio 50:50, v/v) and porcine bile extract (10 mM in the final mixture). Digestion was carried out at 37°C and 150 rpm. Digests samples were withdrawn after 60 min (KD60) and 120 min (KD120) incubation, stopping the enzymatic reaction with Pefabloc® SC (5 mM) and snap freezing. Digestion was performed in duplicate. A digestion blank containing the mixture of enzymes used in digestions at the same concentration without KPC was prepared.

Digests K0, KG120, KD60, and KD120 were subjected to ultrafiltration through a hydrophilic 5000 Da cutoff membrane (Agilent Technologies, Inc., Waldbronn, Germany). Digests and fractions > 5 kDa and < 5 kDa were freeze dried and kept at -20°C until further analyses. The protein content of digests and fractions was determined by the bicinchoninic acid method (BCA) (Pierce, Rockford, IL, USA), using bovine serum albumin as standard protein.

2.4. SDS-PAGE

Samples were dissolved (0.5 mg of protein/mL) in sample buffer that contained Tris-HCl (0.05 M, pH 6.8, Sigma-Aldrich), glycerol (8%, v:v, Panreac Química SAU,

Castellar del Vallés, Barcelona, Spain), sodium dodecyl sulfate (SDS, 1.6 %, w:v, Merck, Darmstadt, Germany), β-mercaptoethanol (2%, v:v, Sigma-Aldrich), and bromophenol blue indicator (0.002%, w:v, Merck). After heated at 95°C for 5 min, 25 µL of samples (12.5 µg protein) were loaded on 12% Bis-Tris polyacrilamide gels (Criterion_XT, Bio-Rad, Hercules, CA, USA). Electrophoretic separations were run at 100 V for 5 min and then, at 150 V for 50 min, using the XT MES running buffer (Bio-Rad) in the Criterion Cell (Bio-Rad). A molecular weight marker (Precision Plus Protein™ Unstained standard, Bio-Rad) containing ten Strep-tagged recombinant proteins (10-250 kDa), including three reference bands (25, 50, 75 kDa) was used. Coomasie Blue (Instant blue, Expedeon, Swavesey, UK) was used to stain the gels, and images were taken with a Molecular Imager® VersaDoc™ MP 5000 system (Bio-Rad) and processed with Quantity One®1-D analysis software (Bio-Rad).

2.4. Measurement of biological activities

2.4.1. Angiotensin I-converting enzyme (ACE) inhibitory activity

In vitro ACE inhibitory activity of digests and fractions was measured using the method described by Sentandreu and Toldrá,[21] and modified by Quirós et al.[22] This method is based on the hydrolysis of the internally quenched fluorescent substrate Abz-Gly-p-Phe(NO_2)-Pro-OH by the action of ACE. The fluorescence of the samples was measured at excitation and emission wavelengths of 350 and 420 nm, respectively, in a FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany) with the FLUOstar (version 1.32 R2, BMG Labtech Inc.) control system for processing of the data. The activity was expressed as IC_{50} or protein/peptide concentration required to inhibit the original ACE activity by 50%, and was determined in duplicate.

2.4.2. Oxygen radical absorbance capacity (ORAC)

An oxygen radical absorbance capacity (ORAC)-FL assay was used following the optimized protocol of Hernández-Ledesma and coworkers.[23] Briefly, the final assay mixture (200 µL, pH 7.4) containing FL (70 nM), AAPH (14 µM), and antioxidant Trolox (0.2-1.6 nmol) or sample (at different concentrations) was incubated at 37°C. Fluorescence was recorded during 137 min (104 cycles) in a FLUOstar OPTIMA plate reader (BMG Labtech Inc.) with 485 nm excitation and 520 nm emission filters. The equipment was controlled by the FLUOstar Control version 1.32 R2 software for fluorescence measurement. Final ORAC-FL value was expressed as µmol Trolox equivalents per mg protein or peptide. Three independent runs were performed for each sample.

2.4.3. Colon cancer cell viability inhibitory activity

Human colorectal cancer Caco-2 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in Dulbecco´s Modified Eagle Medium (DMEM, Biowest, Nuaillé, France), supplemented with 10% (v:v) fetal bovine serum (FBS, Biowest), 1% (v:v) non-essential amino acid solution (Lonza Group Ltd), and 1% (v:v) penicillin/streptomycin/amphotericin B solution (Biowest). The cells were maintained in an incubator under a 5% CO_2/95% air at 37°C and constant humidity. Culture medium was changed every two days, and cells were kept sub-confluent by using trypsin/EDTA (Lonza Group Ltd).

The effect of KPC digests and fractions on the viability of Caco-2 cells was evaluated by the MTT assay. Cells were seeded at a density of 5×10^4 cells/well in 96-well plates (Costar, Corning, NY, USA) and incubated for 24 h. Afterwards, cells were treated with KPC digests, fractions or blanks at different concentrations ranged from

0.05 to 4 mg protein/mL for 24 h. Culture medium was removed, and cells were washed with PBS and incubated with a MTT solution (0.5 mg/mL, final concentration) for 2 h. After discarding the supernatants, insoluble formazan crystals formed were dissolved in DMSO:ethanol (1:1, v:v) and the absorbance was measured at 570 nm in a FLUOstar OPTIMA plate reader (BMG Labtech Inc.). Results were expressed as IC_{50} or protein/peptide concentration needed to inhibit 50% cell viability. Experiments were carried out in triplicate.

2.4.4. In vitro anti-diabetic activity

DPP-IV inhibitory activity was measured in 96-well plates following the protocol described by Silveira et al.[24] Recombinant soluble human DPP-IV (0.26 mU per test well; 15 μL) was incubated at 37ºC in the absence or presence of different concentrations of samples (final volume 50 μL per well) for 10 min. A volume of 50 μL of the assay buffer containing H-Gly-Pro-*p*-nitroaniline was added to each well at final concentration of 100 μM. Absorbance was read at 405 nm in a microplate reader (BMG Labtech Inc.) for 30 min at 2 min time intervals. The results were expressed as IC_{50} value or protein/peptide concentration needed to inhibit 50% of DPP-IV activity. Each sample was analyzed in duplicate.

α-amylase inhibitory activity was determined following the protocol described by Johnson et al.[25] with some modifications. Briefly, 50 μL of sample, positive control (2 mM acarbose) or negative control (distilled water) were added to 100 μL α-amylase solution (2 U/mL in 0.02 M sodium phosphate buffer, pH 6.9). Test tubes were incubated at 20°C in a thermomixer (Eppendorf Iberica, Madrid, Spain) for 5 min. A volume of 100 μL of 1% potato soluble starch solution were added to each tube and incubated at 20ºC, 1,000 rpm for 6 min. Finally, 100 μL of dinitrosalicylic acid color

reagent were added, and the tubes were placed in a 100°C water bath for 15 min. A volume of 800 µL of distilled water was added to the mixture, and the absorbance was read at 540 nm in a Synergy HT microplate reader (Biotek, Winooski, VT, USA). Percent inhibition was calculated relative to the negative control having 100% enzyme activity. α-amylase inhibitory activity was expressed as the peptide concentration needed to inhibit 50% of enzymatic activity (IC_{50}). IC_{50} values were determined by dose-response curves in which the range of concentrations was distributed in a logarithmic scale and using the nonlinear regression sigmoidal curve fit function in GraphPad Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA).

2.5. Fractionation of the kiwicha protein digest by semi-preparative RP-HPLC

Semi-preparative RP-HPLC was carried out according to Vilcacundo et al.[26] with some modifications. A Hi-Pore Reversed Phase RP-318 (250 x 21.5 mm) column (Bio-Rad) was used. Digest KD60 was prepared at a concentration of 10 mg/mL, and the injection volume was 400 µL. Fractions were eluted at a flow rate of 10 mL/min, with a linear gradient of solvent B (acetonitrile: trifluoroacetic acid (TFA), 1000:0.8, v/v) in A (water:TFA, 1000:1, v/v) going from 0% to 70% B in 40 min, 70% to 100% B in 15 min, 15 min with 100% B and from 100% B to 0% B in 20 min. Each chromatographic run was repeated 50-55 times, and the fractions were collected automatically with a Fraction Collector (Model II, Waters, Mildford, MA, USA). The collected fractions were pooled, frozen, lyophilized, and stored at -20 °C until further analyses. The peptide content of collected fractions was determined by the BCA method.

2.6. Peptide identification by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis was performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik GmbH, Bremen, Germany), and equipped with an electrospray ionization source. The column used was a reverse phase Mediterranea Sea C_{18} Column (150×2.1 mm i.d., 5 μm particle size) (Teknokroma, Barcelona, Spain). Peptides were eluted with a linear gradient of solvent B (acetonitrile:formic acid, 1000:1, v/v) in A (water:formic acid, 1000:1, v/v) going from 0% in 45% in 60 min at a flow rate of 0.2 mL/min. The injection volume was 50 μL. Using Data Analysis™ (version 3.0; Bruker Daltonics), the *m/z* spectral data were processed and transformed to representing mass values. For peptide sequencing, the matched MS/MS spectra were interpreted by using BioTools (version 2.1; Bruker Daltonics), and MASCOT from Matrix Science (Boston, MA, USA), using a homemade database that includes the main sequenced proteins of amaranth.

2.7. Statistical analysis

Data represent the mean and standard deviation of two or three independent experiments. Data were subjected to one-way analysis of variance (ANOVA) to compare experimental values using Statgraphics 5.0 (Statistical Graphics Corporation, Rockville, Md). Comparison between groups was performed using a Duncan's multiple-range test, and differences were considered significant at $P \leq 0.05$.

3. Results and Discussion

3.1. Effect of gastrointestinal digestion on the biological activity of kiwicha proteins

The protein concentration of KPC was 53.59%, similar to that (53.60%) reported by Escudero and coworkers, using whole *A. cruentus* flour and pH 11.0 in the extraction step.[27] KPC was subjected to an *in vitro* gastrointestinal digestion simulating physiological conditions. Protein patterns obtained by SDS-PAGE of KPC at the starting point of the digestion (K0), the gastric digest after 120 min incubation with pepsin (KG120), and gastroduodenal digests obtained after 120 min incubation with pepsin and 60 min (KD60) and 120 min (KD120) incubation with pancreatin are shown in Figure 1. The protein profile of KPC (lane 1) and K0 (lane 2) showed bands with molecular weights between 6.5 and 150 kDa with the major bands around 78, 55, 30-35, 20-25, 17, 15, 13, and 11 kDa. This profile is in agreement with previous studies on the electrophoretic pattern of amaranth proteins. According to Gamel et al.,[28] the albumins show three bands, two between 42 and 25 kDa and one below 20 kDa, while the globulins show major bands at 78 and 54 kDa, two bands between 40 and 38 kDa, three bands between 27 and 21 kDa and two bands at 15 and 14 kDa. Glutelins show two bands between 67 and 43 kDa, and two bands between 38 and 35 kDa, while prolamine bands are found between 34 and 20 kDa. During gastric phase, amaranth proteins were partially hydrolyzed by pepsin, the intensity of bands over 25 kDa being negligible, although bands at 25 and 20 kDa could be noticed (lane 3 in Figure 1). The complete protein degradation was observed after their incubation with pancreatin (lanes 4 and 5). Bands appearing at the gel corresponded to enzymes used in simulated digestive process since they were also visible when digestion blank was analyzed (lane 6). Low molecular weight bands were barely detected in KD60 and KD120 digests. That could be due to an extensive proteolysis and production of small peptides that leached out from the gel.

Gastric and gastroduodenal digests from KPC were analyzed for the potential multifunctionality of peptides released during the simulated digestion. The antioxidant capacity of these digests as well as their ability to inhibit ACE, DPP-IV, α-amylase, and colon cancer cell viability were measured (Table 1). In the case of antioxidant capacity, the highest ORAC value was determined in the digest KD60 compared with values obtained for KG120 and KD120. This result indicates that during the first hour of the duodenal phase, potent peroxyl radical scavenging peptides were released. The key impact of gastrointestinal digestion on the release of antioxidant peptides from parent proteins as well as on the modification or breakdown of peptides with antioxidant properties has been recently reported in other plant proteins.[29] Orsini Delgado et al. determined the antioxidant capacity of amaranth proteins digested under *in vitro* gastrointestinal conditions.[30] The ORAC values obtained by these authors (ranged from 0.80 to 1.16 μmol TE/mg protein) were lower than those found in our study. The differing amaranth species and/or digestion conditions might have caused the release of less active protein hydrolysis products.

At the starting point of digestion, K0 showed ACE-inhibitory activity with an IC_{50} value of 79.13 ± 1.08 μg protein/mL (data not shown). Fritz and coworkers had also found ACE inhibition for the non-hydrolyzed isolate from *Amaranthus mantegazzianus*, suggesting the presence of preexisting ACE inhibitory peptides in the protein concentrate.[31] However, in a previous study, Tiengo et al. had reported the practical inactivity of intact amaranth proteins since the IC_{50} value determined for the protein concentrate extracted from defatted *A. cruentus* flour was in the mg scale (12 mg protein/mL).[32] During the gastric phase, IC_{50} value was reduced to 39.0 ± 3.0 μg protein/mL, In the gastroduodenal digests, the inhibition rate returned to the initial values (81.0 ± 10.5 for KD60 and 88.0 ± 14.0 μg protein/mL for KD120), indicating

that some ACE-inhibitory peptides released by pepsin were degraded by the action of pancreatin. The inhibition was superior to that found in *A. cruentus* protein concentrate (439.0 ± 18 μg protein/mL) digested under simulated conditions.[32] Recently, globulin and albumin fractions extracted from *A. hypochondriacus* protein and hydrolyzed with Alcalase were analyzed for their ACE inhibitory activity.[33] These authors reported IC_{50} values between 925 and 1887 μg/mL. Therefore, the Alcalase hydrolysis favored the promotion of ACE inhibitory activity to a less extent than simulated digestion. Lower ACE inhibitory activity was also found for digestion products of soybean protein isolate (IC_{50} of 0.28 mg/mL[34]), and defatted lupine flour protein (IC_{50} from 0.21 to 0.33 mg/mL[35]).

In vitro anti-diabetic properties of KPC and the corresponding digests were also analyzed. Undigested KPC at a concentration of 6.0 mg of protein/mL did not show inhibitory effects against DPP-IV and pancreatic α-amylase (data not shown). After the gastric phase, the inhibitory activity against DPP-IV was observed, although no effects against α-amylase at the highest concentration used (4 mg/mL) were shown (Table 1). Once the gastric digest was subjected to the action of pancreatin, potent DPP-IV inhibitory activity, with similar IC_{50} values of 0.32 ± 0.01 mg protein/mL (KD60) and 0.28 ± 0.01 mg protein/mL (KD120), was developed. These values were lower than those reported by Nongonierma & Fitzgerald[36] for *in vitro* simulated digests obtained from hemp, pea, rice and soy proteins (IC_{50} values between 1.85 ± 0.34 and 4.50 ± 0.55 mg dry weight hydrolyzate/mL) although comparable to those reported for quinoa protein peptides released under the same *in vitro* gastrointestinal digestion conditions.[26] Velarde-Salcedo et al. also determined the DPP-IV inhibitory activity of amaranth peptides released after simulated gastrointestinal digestion, showing an IC_{50} of 1.1 mg/mL.[16] α-amylase inhibitory potency of duodenal digests increased as function of

time as indicated the significantly *($p < 0.05$)* higher IC_{50} value found in KD60 compared to KD120 (Table 1). The α-amylase inhibitory activity of peptides released during the duodenal phase of kiwicha protein digestion was within the range reported for isolated peptides from cumin seed (IC_{50} =1.58-5.6 mg/mL),[37] pinto bean Protamex hydrolyzates (45-55% inhibition at 16.6 mg/mL), and their isolated peptides (IC_{50}=0.5-5.43 mg/mL).[38,39] As compared to our previous study in quinoa, peptides formed during kiwicha protein digestion showed lower α-amylase inhibitory activity compared to quinoa gastroduodenal digests at 60 (IC_{50}=1.75 mg protein/mL) and 120 min of pancreatin hydrolysis (IC_{50}=1.45 mg protein/mL).[26]

In the present study, the potential cytotoxicity in the gastrointestinal tract was evaluated by the MTT protocol, adding the KPC digests to the culture medium after the cells had grown for 24 h, as it has been previously described.[40] Caco-2 cells were selected to study the cytotoxicity of KG120, KD60, and KD120 digests. Digestion blanks without KPC were assayed every time to evaluate the effect of digestive enzymes and bile salts on cell viability. KG120 showed cytotoxic effects in a dose-dependent manner with a maximum inhibitory activity (80.15%) observed when Caco-2 cells were exposed to 4 mg protein/mL of digest, while its corresponding digestion blank did not show cytotoxic effects at this concentration. This result suggests that peptides released by the action of pepsin could be responsible for the observed effects. The cell viability inhibitory activity of gastroduodenal digests significantly increased (between 5- and 9-times) in comparison with that shown by gastric digest, indicating that more potent peptides inhibiting Caco-2 cell viability were released during the first 60 minutes of incubation with pancreatin. However, the contribution on the activity of this extract, bile salts or Pefabloc added to the digestion blank cannot be discarded. The corresponding blanks showed an IC_{50} value of 1.24 ± 0.04 mg/mL.

In order to determine the influence of the molecular weight of peptides on the multifunctionality of kiwicha protein digests, KD60 and KD120 were separated into > 5 kDa and < 5 kDa molecular weight peptide fractions, (Table 1). Low molecular weight peptides seem to be the main responsible for the antioxidant, ACE, DPP-IV, and α-amylase inhibitory activities of gastroduodenal digests.

Smaller molecular weight peptides from both animal and vegetal protein sources have been found to have higher antioxidant activity.[41,42] In our study, fraction < 5 kDa of KD60 showed the highest peroxyl radical scavenging capacity with an ORAC value of 3.02 ± 0.13 μmol TE/mg protein, that meant 70.5% of the activity shown by the whole digest. A slight antioxidant activity was measured in the fraction > 5 kDa from this gastroduodenal digest, supporting the hypothesis that the antioxidant capacity of food derived peptides is not only related to their size but also to their amino acid composition, structure, and hydrophobic character, that determines the mechanism and efficiency of the antioxidant peptides.[43] Also, the ultrafiltration method carried out to separate both peptide fractions could allow the passage of low molecular weight active peptides to fraction > 5 kDa that contributed on the observed effects in this fraction.

Similarly, ACE-inhibitory activity of fractions enriched in short peptides was similar to that determined in the whole digests but the > 5kDa fraction showed a significant activity decrease. This is in agreement with previous studies that have described the ACE-inhibitory activity to be associated with low molecular weight peptides.[44] As an example, fractionation of thermolysin cherry seed protein hydrolyzate by ultrafiltration membrane showed that the smallest peptide fraction (< 3 kDa) was the most active ACE inhibitor when compared to the > 3 kDa peptide fraction.[45]

In contrast, no substantial changes in the DPP-IV inhibitory activity derived from the molecular weight fractionation were observed. Though, the low value

determined in the fraction < 5 kDa at the end of simulated digestion (KD120) merits attention because it points to an increased recovery of potent inhibitory peptides at the end of the gastrointestinal digestion. On the other hand, only fractions < 5 kDa showed inhibition of pancreatic α-amylase, although the IC_{50} values were lower in the 120 min digest. These results suggested that potent α-amylase inhibitory peptides from kiwicha proteins are mainly generated at the end of the gastroduodenal digestion. Similarly, previous studies have established low molecular weight peptides of < 3-5 kDa as the main α-amylase inhibitors in pinto bean Protamex hydrolyzate,[38] and quinoa gastroduodenal digests.[26]

Fractions > 5 kDa and < 5 kDa obtained from KD60 and KD120 were also assayed for their cytotoxic effects against Caco-2 cells (Table 1). Between ultrafiltration fractions, > 5 kDa peptides showed higher potency to inhibit Caco-2 cell viability than < 5 kDa peptides. The fraction containing short peptides obtained from KD60 did not show any activity at the highest concentration assayed (2.5 mg protein/mL). Digestive enzymes retained in fraction > 5 kDa and bile salts that passed through the membrane filter appearing in fraction < 5 kDa could be responsible for the cytotoxic effects shown by fractions collected from the digestion blank. Our results indicate that high molecular kiwicha peptides were more effective inhibiting colon cancer cell proliferation than smaller peptides. This is in agreement with González-Montoya et al.[46] who found high molecular fractions collected from germinated soybean digests as the most active inhibiting human colon cancer cells viability. However, Kannan and coworkers found the < 5 kDa fraction of rice bran peptide hydrolyzates to have higher anti-cancer activity against HepG-2 and Caco-2 cells than both 5-10 and >10 kDa fractions.[47] Although the greater molecular mobility and diffusivity of low molecular weight peptides has been considered to facilitate their interactions with cancer cell components improving their

antiproliferative activity,[48] for our kiwicha digests, other aspects such as the amino acid composition and the peptide hydrophobicity could also have a positive impact on the activity of > 5 kDa fraction.

3.2. Identification of potential bioactive peptides

To identify and characterize the bioactive peptides released during gastroduodenal digestion of kiwicha proteins, KD60 was selected based on its multifunctional potential, and fractionated by semi-preparative RP-HPLC (Figure 2). Most of the kiwicha peptides contained in the gastroduodenal digest eluted between 30 and 50 min. Two fractions, named as F-1 and F-2, were collected and analyzed to determine their biological activities (Table 2). Both fractions were found to exert all the bioactivities studied, with F-2 being more active except for α-amylase inhibition that showed an IC_{50} value 2.8-times higher than F-1. The ORAC value of F-2 was 4.47 µmol TE/mg protein compared to 1.56 µmol TE/mg protein calculated for F-1. The IC_{50} values for ACE, DPP-IV and Caco-2 cell viability inhibition shown by F-2 were between 1.3- and 4.4-fold lower than those determined for F-1. Both fractions were analyzed by LC-MS/MS in order to identify the peptides present in them and potentially responsible for their biological activity. With this analysis, diverse peptide compounds with molecular mass in the range 500 to 1500 Da were detected. Due to incomplete knowledge of the *Amaranthus* genome, only some amaranth seed proteins have a reported sequence included in the NCBInr database. Thus, only peptides belonging to those referenced proteins can be identified by this approach. In our study, 13 fragments matched amaranth proteins (11S globulin, acetylate synthase, albumin 1, and polyamine oxidase) found in the database, of which five were present in F-1 and eight in F-2 (Table 3). Of these identified peptides, sequences NRPET, HVIKPPS, and

ASANEPEDEN, were within the sequence of peptides (FNRPETT, HVIKPPSRA, and ITASANEPEDN) recently identified by Orsini Delgado et al.[18] These authors showed an ORAC IC_{50} value of 0.57 mg/mL for peptide HVIKPPSRA. The quantitative structure activity relationship (QSAR) modeling has demonstrated the relationship between the physicochemical properties at the C-terminal and N-terminal regions and the antioxidant potency, the former being more relevant than the latter. Bulky hydrophobic amino acids at the C-terminal region, polar/charged amino acids at the C1 position as well as low electronic property at the N1, N2 positions have also been found to contribute to the antioxidant activity.[49] Hernández-Ledesma et al. evaluated the ORAC activity of each amino acid, showing that tryptophan, tyrosine, methionine, histidine, and phenylalanine showed peroxyl radical scavenging activity whereas the rest of amino acids had no activity.[23] Leucine has also been reported to enhance the scavenging activities of peptides.[50] In our case, peptides containing bulky amino acids, and mainly the peptide containing tyrosine might be responsible for the antioxidant capacity observed.

The ACE-inhibitory activity of long-chain (> 4 residues) seems to be mainly determined by the C-terminal tetrapeptide.[51] Moreover, the presence of proline, branched-chain, aromatic, or hydrophobic amino acids in these peptides seem to enhance their inhibitory properties.[44] Of peptides released from kiwicha proteins during the intestinal phase, eight contain more than two leucine, proline, valine, or histidine residues. Moreover, among them, sequences FLISCLL, SVFDEELS, and DFIILE contained phenylalanine that could also be determinant on their ACE-inhibitory potency.

Proline at the first, second, third, or fourth N-terminal position has been defined as a structural feature determinant on the activity of DPP-IV inhibitory peptides.[52]

Moreover, recent *in silico* studies have shown that potent DPP-IV inhibitory peptides generally contain a branched-chain amino acid (leucine, isoleucine, or valine) or an aromatic residue with a polar group in the side-chain (primarily tryptophan) at their N-terminal position.[53,54] On basis of these studies, peptides NRPET and VEEGNM, released from 11S globulin and albumin 1, respectively, could be the main responsible for the DPP-IV inhibitory activity shown by kiwicha fractions. However, other peptides contained in these fractions that have not been identified as well as their potential interactions might also have a positive influence.

Recently, it has been reported that the aromatic-aromatic interactions (hydrogen bonds, electrostatic and Van der Waals interactions) between food-derived peptides and enzyme residues in the catalytic site seem to be crucial for the inhibition of α-amylase activity.[55] Therefore, the presence of aromatic residues such as phenylalanine, tryptophan, and tyrosine has been established as structural feature of peptides for α-amylase inhibition.[39,55] Previously, peptides derived from pinto bean proteins and containing histidine, methionine, leucine, proline, or glycine had been found good inhibitors of α-amylase.[38,56] Most of peptides identified in F-1 (YESGSQ, GGEDE, and NRPET) and F-2 (FLISCLL, TALEPT, HVIKPPS, SVFDEELS, ASANEPDEN, and DFIILE) contained the amino acid residues involved on enzyme binding at N-terminal and C-terminal of the peptide sequence. Position of these amino acids in the peptide sequence also plays an important role on α-amylase inhibition.[38]

Taken together all results and on the basis of the relationship between the structure and bioactivities, peptides identified in fraction F-2 (FLISCLL, SVFDEELS, and DFIILE) have a great potential as multifunctional peptides exerting antioxidant capacity and ability to inhibit both ACE and α-amylase. Peptide NRPET (F-1) as

inhibitor of DPP-IV and α-amylase, and peptide HVIKPPS (F-2) as antioxidant and α-amylase inhibitor might also be considered as multifunctional peptides.

The results of the present study demonstrate the role of kiwicha proteins as source of multifunctional peptides released under *in vitro* conditions simulating gastrointestinal digestion. After sequential incubation with pepsin and pancreatin, potent digests with antioxidant capacity and inhibitory activity against ACE, DPP-IV, and colon cancer cell viability were obtained. These digests also showed moderate α-amylase inhibitory activity. Peptides contained in these digests might exert their effects at both local and systemic level after their absorption through the gastrointestinal tract. Low molecular weight peptides were the main responsible for the radical scavenging activity and the activity towards enzymes, while higher size peptides were the main determinant on the cytotoxic effects against colon cancer cells. Thirteen peptides have been identified of which five show high potential to exert multifunctional properties. Thus, kiwicha proteins might start to gain importance as ingredients for functional foods for the prevention and/or management of chronic diseases related to oxidative stress, hypertension and/or diabetes. However, the findings of this study should be validated using the individual peptides to confirm their multifunctionality and investigate their bioavailability and mechanism of action.

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Abbreviations used

AAPH, 2,2’-azobis (2-methylpropionamide)-dihydrochloride; ACE, angiotensin-converting enzyme; ATCC, American Type Culture Collection; BCA, bicinchoninic acid; DMEM, Dulbecco´s Modified Eagle Medium; DMSO, dimethylsulfoxide; DPP-IV, dipeptidyl peptidase IV; FBS, fetal bovine serum; FL, fluorescein disodium; KPC, kiwicha protein concentrate; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide; ORAC, oxygen radical absorbance capacity; RP-HPLC-MS/MS, RP-HPLC coupled to tandem mass spectrometry; SDS, sodium dodecyl sulphate; TFA, trifluoroacetic acid; Trolox, (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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Figure captions

Figure 1. Characterization of the kiwicha protein digests obtained after an *in vitro* simulated gastrointestinal digestion by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. MW: molecular weight marker; Lane 1: kiwicha protein concentrate (KPC), Lane 2: KPC digest at time 0 (K0), Lane 3: KPC gastric digest at 120 min (KG120), Lane 4: KPC gastroduodenal digest at 60 min (KD60), Lane 5: KPC gastroduodenal digest at 120 min (KD120), Lane 6: Digestion blank with digestive enzymes.

Figure 2. Fractionation by preparative RP-HPLC of the kiwicha protein concentrate (KPC) gastroduodenal digest at 60 min (KD60). Collected fractions are termed with F followed by a number.

Table 1. Antioxidant activity and ACE, DPP-IV, α-amylase, and colon cancer Caco-2 cells viability inhibitory activities of gastric and gastroduodenal digests of kiwicha protein concentrate and their fractions > and < 5 kDa

Digest	Fraction	Antioxidant activity (ORAC, µmol TE/mg protein)	ACE inhibition (IC_{50}, µg protein/mL)	DPP-IV inhibition (IC_{50} – mg protein/mL)	α-amylase inhibition (IC_{50} – mg protein/mL)	Caco-2 cell viability inhibition (IC_{50} – mg protein/mL)	
						Blank	Sample
KG120	Whole digest	1.51 ± 0.01[b]	39.00 ± 2.99[a]	0.66 ± 0.01[d]	n.d.[+]	n.d.[+]	0.72 ± 0.01[e]
KD60	Whole digest	4.28 ± 0.11[e]	81.00 ± 10.53[b]	0.32 ± 0.01[b]	2.73 ± 0.02[d]	1.24 ± 0.04[g]	0.08 ± 0.002[a]
	F > 5 kDa	0.99 ± 0.03[a]	133.74 ± 2.55[c]	0.46 ± 0.01[c]	n.d.[*]	0.46 ± 0.01[d]	0.33 ± 0.03[c]
	F < 5 kDa	3.02 ± 0.13[d]	75.61 ± 5.77[b]	0.45 ± 0.03[c]	1.81 ± 0.05[b]	0.80 ± 0.01[f]	n.d.[*]
KD120	Whole digest	3.03 ± 0.06[d]	88.01 ± 13.96[b]	0.28 ± 0.01[ab]	2.45 ± 0.01[c]	1.24 ± 0.04[g]	0.14 ± 0.01[ab]
	F > 5 kDa	0.98 ± 0.08[a]	181.85 ± 6.12[d]	0.68 ± 0.07[d]	n.d.[*]	0.46 ± 0.01[d]	0.19 ± 0.01[b]
	F < 5 kDa	2.27 ± 0.12[c]	83.90 ± 6.81[b]	0.19 ± 0.01[a]	0.84 ± 0.03[a]	0.80 ± 0.01[f]	1.76 ± 0.11[h]

[a-g]: Different lowercase letters in each biological activity indicate significant differences among samples (*p*<0.05, Duncan test)
n.d.[+]: No inhibitory effect observed at the highest concentration used (4 mg/mL)
n.d.[*]: No inhibitory effect observed at the highest concentration used (2.5 mg/mL)
TE: Trolox equivalents

Table 2. Antioxidant activity and ACE, DPP-IV, α-amylase and colon cancer Caco-2 cells viability inhibitory activities of fractions collected by RP-HPLC from gastroduodenal digests of kiwicha protein concentrate (KPC) for 60 min (KD60)

Fraction	Antioxidant activity (ORAC, µmol TE/mg protein)	ACE inhibition (IC_{50}, µg protein/mL)	DPP-IV inhibition (IC_{50}, mg protein/mL)	α-amylase inhibition (IC_{50}, mg protein/mL)	Caco-2 cell viability inhibition (IC_{50}, mg protein/mL)
F-1	1.56 ± 0.13^{a}	359.47 ± 0.52^{b}	0.38 ± 0.04^{b}	0.42 ± 0.03^{a}	1.17 ± 0.14^{a}
F-2	4.47 ± 0.39^{b}	81.47 ± 7.61^{a}	0.18 ± 0.01^{a}	1.17 ± 0.05^{b}	0.87 ± 0.08^{a}

$^{a-b}$: Different lowercase letters in each column indicate significant differences among samples (*p*<0.05, Duncan test)

TE: Trolox equivalents

Table 3. Peptides identified by HPLC-MS/MS in the fractions F-1 and F-2 collected from kiwicha protein concentrate (KPC) after gastroduodenal digestion for 60 min (KD60).

Fraction	Observed mass	Calculated mass	Sequence	Fragment	Source protein	NCBI Accession number
F-1	669.30	669.26	YESGSQ	f(130-135)	11S Globulin	CAA57633.1
	505.17	505.16	GGEDE	f(139-143)		
	615.31	615.29	NRPET	f(479-483)		
	614.39	614.34	QQQLV	f(254-258)	Acetylate synthase	AAK50821.1
	517.29	517.22	ACDIP	f(606-610)		
F-2	807.40	807. 45	FLISCLL	f(22-28)	11S Globulin	CAA57633.1
	630.25	630.32	TALEPT	f(56-61)		
	776.52	776.45	HVIKPPS	f(288-294)		
	924.49	924.40	SVFDEELS	f(402-409)		
	945.33	945.36	ASANEPDEN	f(81-89)	Albumin 1	1JLY_B
	677.26	677.26	VEEGNM	f(103-108)		
	748.40	748.40	DFIILE	f(56-61)	Polyamine oxidase	AAM43922.1
	630.24	630.32	EVEAAI	f(133-138)		

Figure 1

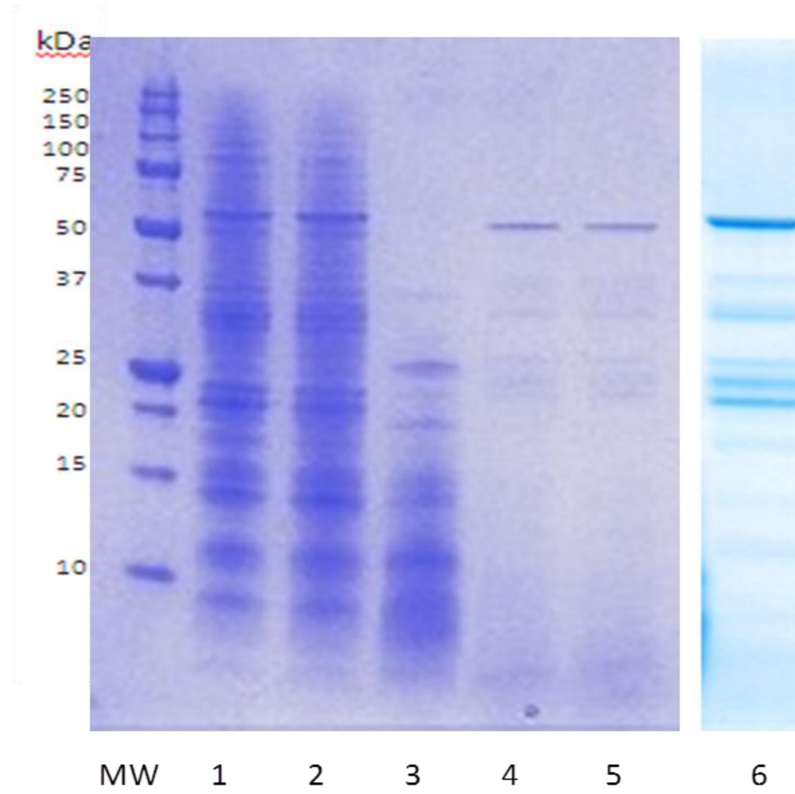
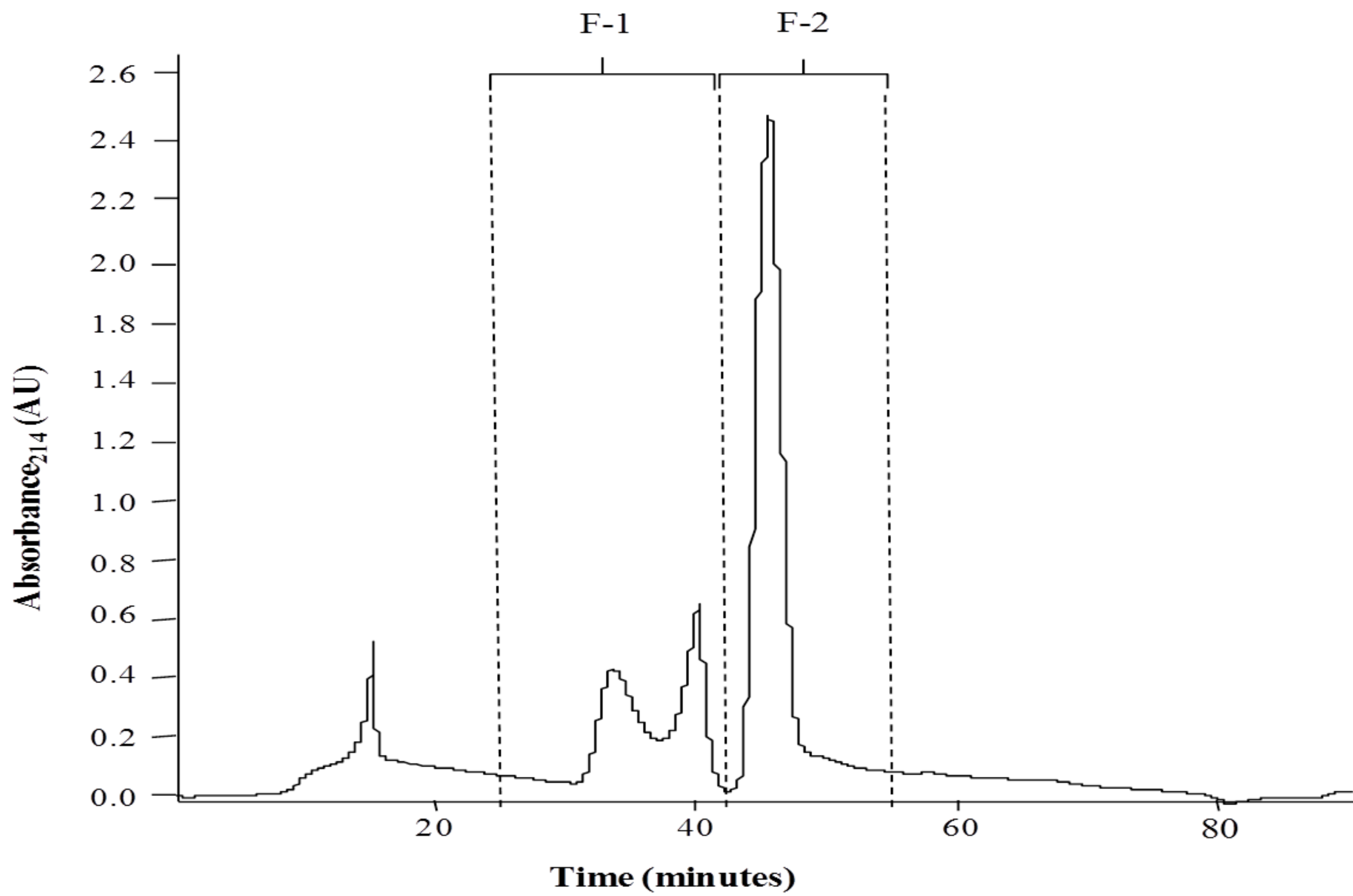


Figure 2



Publicación VII: **Evaluación de la capacidad inhibidora de la peroxidación lipídica de proteína de kiwicha (*Amaranthus caudatus*) digerida bajo condiciones gastrointestinales simuladas.**

R. Vilcacundo, D. Barrio, C. Carpio, B. Hernández-Ledesma, W. Carrillo. Short communication: Evaluation of the lipid peroxidation inhibitory capacity of Kiwicha (*Amaranthus caudatus*) protein digested under simulated gastrointestinal conditions. Journal of Medicinal Food. Enviado para su publicación.

Resumen

Las larvas de pez cebra han sido empleadas en los últimos años como modelo animal para evaluar la actividad antioxidante (inhibición de la peroxidación lipídica) de hidrolizados de proteínas alimentarias, como la proteína del huevo o la quinua. En este estudio, este modelo fue aplicado para evaluar la capacidad antioxidante de un concentrado de proteína de kiwicha digerido bajo condiciones *in vitro* simulando la digestión gastrointestinal. La mayor actividad se observó en los péptidos liberados al final del proceso digestivo, indicando que la degradación completa de las proteínas de este pseudocereal permitió la liberación de secuencias con un elevado potencial antioxidante.

Evaluation of the lipid peroxidation inhibitory capacity of kiwicha (*Amaranthus caudatus*) protein digested under simulated gastrointestinal conditions

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Abstract

Kiwicha protein concentrate was digested under *in vitro* simulated gastrointestinal conditions. Proteins were almost completely hydrolyzed by pepsin at pH of 1.2, while at higher pHs, only partial hydrolysis was observed. During the duodenal phase, no intact proteins were visible, indicating their susceptibility to the simulated digestive conditions. The potent *in vitro* lipid peroxidation inhibitory capacity shown by the gastric and gastroduodenal digests was confirmed in the zebrafish larvae model. Highest activity was shown by peptides contained in the digest obtained at the end of the digestive process.

1. Introduction

Kiwicha (*Amaranthus caudatus*) is a pseudocereal from the Andean region used by the pre-Colombian cultures for centuries. Similarly to other *Amaranth* species, this crop is currently gaining popularity due to its genetic variability, agronomic advantages, and excellent nutritional properties, presenting high protein content (15–17 %, w/w) and digestibility, and good amino acid balance (Lado et al., 2015). In addition to their nutritional value, amaranth proteins have been recently recognized as source of bioactive peptides, defined as inactive fragments into the source protein that once released during gastrointestinal digestion or food processing exert different biological activities. Several peptides have been isolated after their release from *A. hypochondriacus* and *A. mantegazzianus* proteins and characterized by their angiotensin-converting enzyme inhibitory, anti-inflammatory, and antioxidant activities (Fritz et al., 2011; Montoya-Rodríguez & González de Mejía, 2015; Orsini Delgado et al., 2016). However, to our knowledge, no data on bioactive peptides derived from *A. caudatus* can be found in the literature. Therefore, the aim of this work was to evaluate the effect of an *in vitro* simulated gastrointestinal digestion on the release of antioxidant peptides from kiwicha protein concentrate. The lipid peroxidation inhibitory capacity of digests was evaluated by both *in vitro* and *in vivo* using the zebrafish larvae model. This animal model has been recently applied to evaluate the lipid peroxidation inhibitory activity of peptides isolated from hen egg white lysozyme and gastroduodenal digests from quinoa protein concentrate (Carrillo et al., 2016; Vilcacundo et al., 2017).

2. Materials and Methods

The amaranth protein concentrate (APC) was prepared following the protocol of Acosta et al. (2016) with slight modifications. Amaranth flour (Gramolino, Quito, Ecuador) was suspended in water (1:10, w/v) and its pH was adjusted to 8.0. After stirring the suspension for 1 h, it was centrifuged at 4,500 x *g* for 30 min at 25 °C. The pH of the supernatant was adjusted to 4.0 and it was centrifuged at 4,500 x *g* during 20 min at 4 °C. The pellet was dissolved in a small volume of water, neutralized, lyophilized, and kept at -20 °C until further analyses.

APC (5 mg/mL) was subjected to an *in vitro* simulated gastrointestinal digestion. Firstly, gastric phase was carried out at 37 °C for 120 min using simulated gastric fluid (0.35 M NaCl) at different pHs: 1.2, 2.0 and 3.2, and pepsin (4,500 U/mg protein, Sigma-Aldrich, St. Louis, MO, USA) at an enzyme:substrate (E:S) ratio of 2000 U/mg. At the end of gastric digestion, aliquots of gastric digests (GD) were withdrawn inactivating the enzyme by heating at 80 °C for 5 min. The pH of gastric digest obtained at pH 3.2 was adjusted to 7.0, and the intestinal phase started after the addition of 0.125 M bile salt mixture (Sigma-Aldrich), 1 M $CaCl_2$ (7.6 mM final concentration), and pancreatin (E:S of 100 U/mg) dissolved in 20.3 mM Bis-Tris buffer. Intestinal digestion was carried out at 37 °C for 120 min (DD), inactivating the enzyme by heating at 80 °C for 5 min.

SDS-PAGE of APC and its digests was carried out in a Mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, USA) using 4% and 12% polyacrylamide gels (Bio-Rad). Polypeptide bands were stained with Coomassie Brilliant Blue G-250 for 12 h. Relative molecular masses of protein were determined by comparing them with molecular weight markers (Bio-Rad). Characterization was also carried out by RP-

UHPLC using an Agilent 1200 infinity series UHPLC system (Agilent Technologies, Waldbron, Germany). The column used was Zorbax EC C18 (Agilent Poroshell 120, 4.6 x 50 mm x 2.7 µm of particle size). Peptides were eluted at a flow rate of 1.0 mL/min with a lineal gradient of solvent B (acetonitrile:trifluoroacetic acid, 0.270%, v/v) in solvent A (water: trifluoroacetic acid, 0.370 % v/v) going from 0 to 70% in 10 min.

The lipid peroxidation inhibitory activity of APC and its gastroduodenal digests was evaluated by the *in vitro* Thiobarbituric Acid Reactive Substances (TBARS) method. After oxidizing 500 mg of olive oil by heating at 65 °C for 8 days, samples were added at concentration of 2.0 mg/mL and incubated at 30 °C for 24 h. Negative (olive oil without sample) and positive (butylhydroxytoluene, BHT, 0.2 mg/mL) controls were used. A volume of 1000 µL of sample was mixed with 1000 µL of 1% thiobarbituric acid (TBA) solution, and the mixture was heated at 95 °C for 1 h. Then, the absorbance of the final solution was measured at 532 nm using a spectrophotometer (Thermo Scientific Evolution 200, Madison, WI, USA). The antioxidant activity of APC and its digests was also evaluated by the TBARS method in the zebrafish larvae model as previously described by Carrillo et al. (2016). Larvae were incubated in 24-well plates (30 larvae/well) with APC, GD and DD samples at concentration of digests 2.0 mg/mL. Groups of 30 larvae/well in aquarium water were used as controls. Lipid peroxidation was initiated by adding 1000 µL of 500 µM H_2O_2. After incubation at 28 °C for 8 h, H_2O_2 was removed and 500 µL of Tween 0.1% were added. Larvae were mixed and homogenized. A volume of 1000 µL of 1% TBA was added and the solution was heated at 95 °C for 1 h. The absorbance of the final solution was measured at 532 nm. The values of antioxidant activity were expressed as the percentage of inhibition of lipid peroxidation in larvae homogenate as follows: % Inhibition of lipid peroxidation = [1-

(Ab - As)/Ab × 100], where Ab is the absorbance of blank and As is the absorbance of the sample.

Results and Discussion

APC and its gastroduodenal digests were analyzed using SDS-PAGE. As shown in Figure 1, the protein profile of APC (lane 2) showing bands with molecular weights between 6.5 to 50 kDa was very similar to that obtained by Nora Martínez and Añón (1996) for *A. hypochondriacus* proteins, although the band with 78 kDa corresponding to a subunit from 7S globulin was not observed in our study. Bands with molecular weight of ≈45 and ≈25 kDa corresponded to α- and β-peptides of 11S globulin (amarantin) as it has been previously reported by Thanapornpoonpong and coworkers (2008), while the acidic and basic subunits of this protein were visible at 34 and 20 kDa, respectively (Juan et al., 2007). Bands lower than 18 kDa corresponded to albumin components. These albumins were partially resistant to the action of pepsin when kiwicha proteins were incubated with this enzyme at pH 1.2 (lane 3). However, no bands corresponding to globulins were visible indicating that these proteins were sensitive to the action of this gastric enzyme. At higher pHs (2.0 and 3.2), proteins were resistant to gastric digestion (lanes 4 and 5, respectively). After sequential incubation with pepsin at pH 3.2 and pancreatin, no bands were observed in the gel, indicating that during the duodenal phase amaranth proteins were completely degraded by proteases contained in the pancreatin preparation. This result is in agreement with Orsini Delgado et al. (2011) that reported that the diminution of the total bands intensity observed for the gastroduodenal digest from *A. mantegazzianus* protein was due to the release of peptides with molecular weight lower than the detection limit of the gel. Analysis of kiwicha protein digests was also carried out by

RP-UHPLC (Figure 2). Peptides contained in non-digested APC eluted during the first 10 min of analysis with five fractions, named as F1, F2, F3, F4, and F5 clearly identified in the chromatogram (Fig. 2A). The detection of peaks during UHPLC analysis of APC without digesting suggests the presence of peptides resulting from the action of endogenous enzymes. The profile changed after incubation with pepsin with reduction of the intensity of fractions F1 and F2 and modification of the peptides pattern contained in fractions F3-F5 (Figures 2B-2D). It is remarkable that the profile observed for three gastric digests were similar among them and few changes resulted from the digestion with pancreatin.

The lipid peroxidation inhibitory capacity of gastric and gastroduodenal APC digests was evaluated by the *in vitro* TBARS method. As shown in Figure 3A, no activity was observed for water used as negative control while BHT, used as positive control, inhibited TBARS up to 93.4% at 0.2 mg/mL. No activity was observed for non-digested APC (data not shown). However, after incubation with pepsin at pHs of 1.2, 2.0, and 3.2, TBARS was inhibited by 89.8%, 77.5%, and 78.7%, respectively. The higher inhibitory capacity was shown by the digest obtained at the lower pH (1.2), indicating that the most intense hydrolysis of amaranth protein allowed releasing peptides with higher inhibitory potency. Moreover, during the duodenal phase, the antioxidant capacity increased up to 94.9% inhibition, a value higher than that observed for BHT. Recently, the antioxidant activity of peptides released after simulated gastrointestinal digestion of proteins from *A. mantegazzianus* has been described (Orsini Delgado et al., 2015). These authors reported that the strong augmentation in the scavenging activity against peroxyl, hydroxyl radicals and peroxynitrites resulting from the action of these peptides could be due to their capacity to inhibit the initiation or propagation

of radical reactions by being able to act as metal chelators or by donating a hydrogen atom.

The zebrafish larvae model was used as an *in vivo* method to confirm the antioxidant capacity of APC digests (Figure 3B). This activity was determined as the protective effect against lipid peroxidation induced by hydrogen peroxide in the animal model. The inhibition value obtained for BHT was of 90.0% while water and non-digested APC showed no activity. As it had been observed with the *in vitro* method, gastric digest obtained at pH 1.2 showed the highest antioxidant capacity with an inhibition percentage of 67.6%. Digests obtained at pHs 2.0 and 3.2 showed inhibition values of 59.8% and 42.3%, respectively. Newly, the digest obtained after sequential incubation with pepsin and pancreatin was the most active inhibiting TBARS by 77.6%. Low molecular weight peptides released during the two phases of simulated gastrointestinal digestion could be responsible for the highest activity observed.

Conclusions

The results of this study demonstrate, for the first time that kiwicha protein hydrolyzates obtained during an *in vitro* simulated gastrointestinal digestion are capable of inhibiting lipid peroxidation in both *in vitro* and *in vivo* conditions. Therefore, this amaranth specie is a promising source of antioxidant peptides that could exert a preventive effect against oxidative stress-associated disorders. Moreover, these peptides might be used as additive to control the lipid oxidation responsible for deterioration of food during manufacture and storage. Further studies would be needed to identify the peptides responsible for the antioxidant activity, and to study in depth the *in vivo* mechanisms of action.

Conflict of interest the authors declare there is no conflict of interest.

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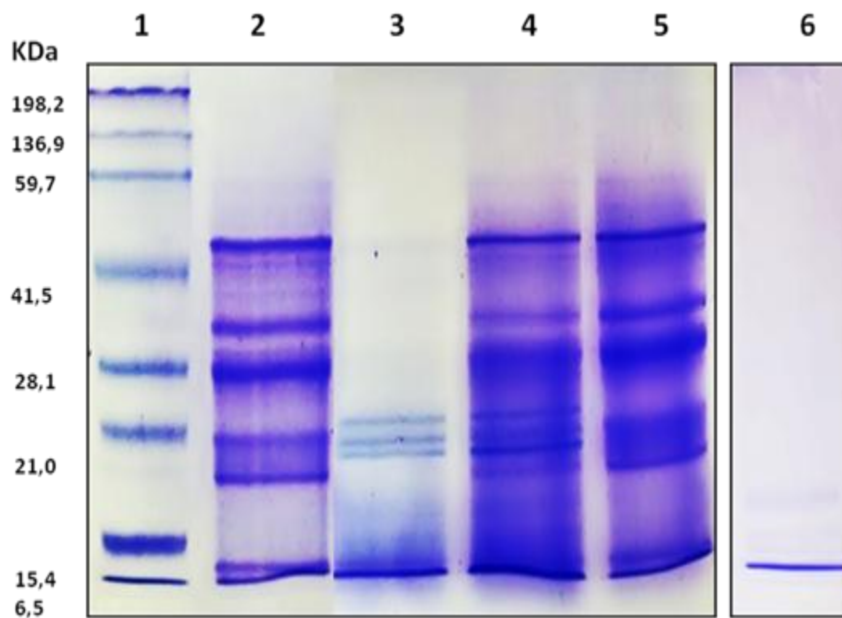

Figure 1. SDS-PAGE electrophoresis analysis of APC and its gastroduodenal digests. 1: molecular weight marker; 2: APC; 3: gastric digest obtained at pH 1.2; 4: gastric digest obtained at pH 2.0; 5: gastric digest obtained at pH 3.2; 6: gastroduodenal digest obtained after incubation with pepsin at pH 2.0 and pancreatin at pH 7.0

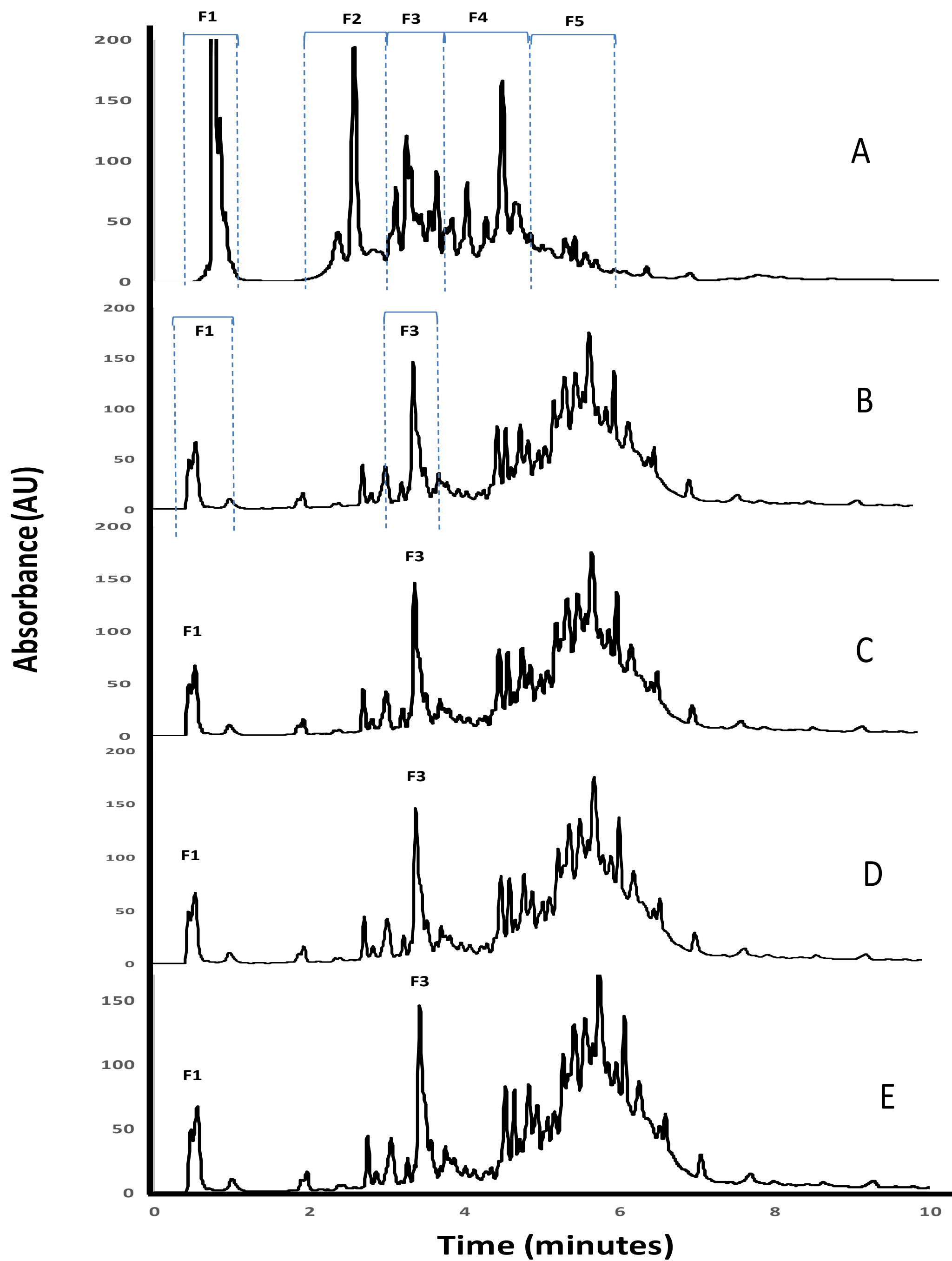

Figure 2. RP-UHPLC analysis of APC and its gastroduodenal digests. (A) APC, (B) gastric digest obtained at pH 1.2 (C) gastric digest obtained at pH 2.0 (D) gastric digest obtained at pH 3.2 (E) gastroduodenal digest

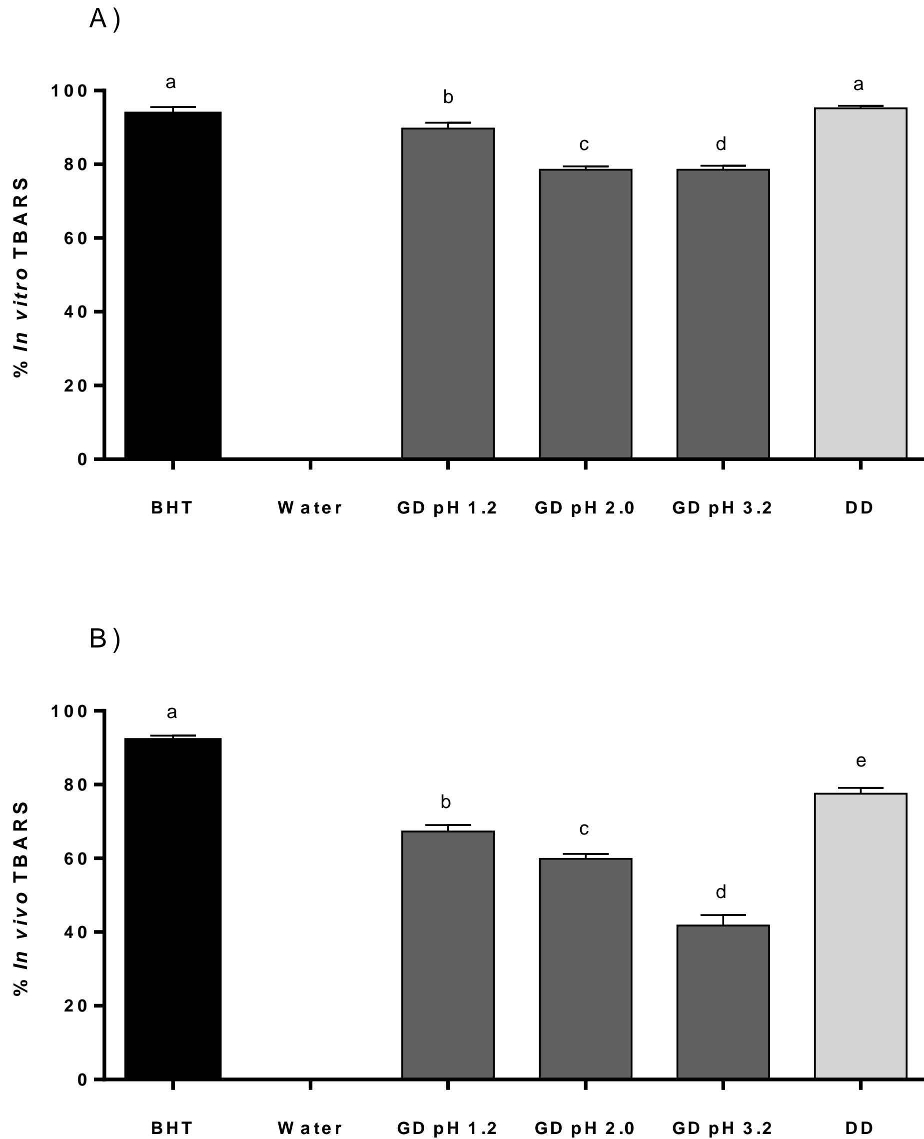

Figure 3. Lipid peroxidation inhibitory activity of gastroduodenal digests from APC. A) *In vitro* TBARS inhibitory capacity, B) TBARS inhibitory capacity evaluated in the *in vivo* zebrafish larvae model. BHT: positive control, water: negative control, GD pH 1.2: gastric digest obtained at pH 1.2, GD pH 2.0: gastric digest obtained at pH 2.0, GD pH 3.2: gastric digest obtained at pH 3.2; DD: gastroduodenal digest

3. DISCUSIÓN GENERAL

La necesidad de garantizar la seguridad alimentaria, el incremento mundial del consumo de proteínas de alto valor biológico, la preocupación por la salud y la utilización eficiente de los recursos del planeta sustentan el desarrollo de ingredientes y alimentos a base de proteínas agroalimentarias alternativas. De hecho, en los últimos años, se ha incrementado el interés por el desarrollo de ingredientes y alimentos basados en productos vegetales poco explotados o bien que se hayan empleado con otros fines, como la alimentación animal o la producción de biocombustibles.

La quinua (*Chenopodium quinoa* Willd.) y el amaranto (*Amaranthus* sp.) son cultivos prehispánicos de gran importancia que formaron parte de la dieta de los Mayas, Aztecas, Incas y otras civilizaciones precolombinas. Son considerados como cultivos de amplia variabilidad genética y alta capacidad adaptativa a diferentes hábitats agroalimentarios y tipos de suelo, además de tener un bajo coste de producción. Estas características hicieron que la FAO, en el año 1996, reconociera a ambos pseudocereales como cultivos que permiten garantizar la seguridad y soberanía alimentaria para la humanidad. Además, la NASA incluyó la quinua en su programa "Controlled Ecological Life Support Systems (CELLS)", siendo seleccionada para su uso en los viajes espaciales de larga duración por sus características y calidad nutricional, su tamaño y su rápido ciclo de vida (Nascimiento y col., 2014). La quinua y el amaranto se caracterizan por un excelente perfil nutritivo con altos porcentajes de proteínas, ácidos grasos, carbohidratos y fibra. Es de destacar el mayor contenido en proteínas de estas dos especies en comparación con el contenido presente en la mayoría de los cereales. El excelente balance de aminoácidos de estas proteínas le confiere una calidad comparable a la de las proteínas de origen animal, como las de la leche de vaca y de huevo de gallina. Además, las proteínas de quinoa y amaranto destacan por su alto grado de digestibilidad (Pérez-Conesa y col., 2002; Escudero y col., 2014).

El género *Chenopodium* tiene una amplia distribución mundial, con 250 especies, siendo *Chenopodium quinoa* Willd. la más utilizada con fines alimentarios. Existe una gran diversidad de productos elaborados a partir de la quinoa como harinas, fideos, hojuelas, granolas y barras energéticas. Sin embargo, otros productos más

elaborados o cuya producción requiera del uso de tecnologías más avanzadas todavía no han sido completamente caracterizados. Además, los estudios existentes sobre los beneficios para la salud de esta planta y concretamente de sus proteínas son muy limitados. El género *Amaranthus* está constituido por unas 70 especies distribuidas en diferentes partes del mundo. Tres especies, *A. hypochondriacus* (México), *A. cruentus* (Guatemala) y *A. caudatus* (América del Sur) se emplean con fines alimentarios y su producción y consumo se ha extendido a lo largo del planeta en los últimos años (Casarrubias-Castillo y col., 2014). A pesar del mayor consumo de la especie andina *A. caudatus*, esta es una de las menos estudiadas en relación a su potencial beneficioso para la salud.

Conocer los procesos que tienen lugar desde la ingesta del alimento hasta el efecto biológico de los compuestos alimentarios es fundamental para establecer la relación entre dicho alimento y la salud. En el caso de las proteínas, un seguimiento de los cambios ocurridos durante la digestión, a través del análisis, tanto de la degradación proteica, como de la generación de secuencias peptídicas es esencial. Los datos obtenidos en humanos son la referencia en estos estudios. Sin embargo, por razones prácticas y éticas, es necesario disponer de modelos *in vitro* de la digestión gastrointestinal. Estos modelos son ampliamente utilizados y se ha demostrado su reproducibilidad y consistencia. Sin embargo, la alta variabilidad de protocolos existentes propulsó la investigación en esta área y a través de la Acción COST FA 1005 “Improving Health Properties of Food by Sharing our Knowledge on the Digestive Process” se ha desarrollado un modelo consensuado a nivel internacional.

La presente Tesis Doctoral se ha centrado en el estudio de las proteínas de quinua (*Chenopodium quinoa* Willd.) y amaranto (*Amaranthus caudatus*) como fuente de péptidos bioactivos tras un proceso de digestión gastrointestinal simulando condiciones fisiológicas. En los concentrados proteicos y los digeridos gástricos e intestinales se llevó a cabo el estudio del perfil proteico y peptidómico mediante electroforesis en gel de poliacrilamida con agentes desnaturalizantes (PAGE-SDS) y se estudiaron diferentes actividades biológicas, empleando modelos *in vitro* e *in vivo.* Se estudió la actividad antioxidante con los métodos *in vitro* ORAC y TBARS, la actividad

inhibidora de las enzimas DPP-IV, α-amilasa y α-glucosidasa relacionadas con la diabetes, la actividad inhibidora de la ECA relacionada con el control de la tensión arterial y la actividad anti-proliferativa, empleando diferentes líneas celulares de cáncer de colon (Caco-2, HT-29 y HCT-116). Tras el fraccionamiento y análisis de las fracciones, los péptidos potencialmente responsables de las actividades biológicas fueron identificados. Los trabajos experimentales incluidos en esta Tesis revelan nuevos datos que sugieren la potencialidad de las proteínas de quinua y amaranto como fuente de péptidos bioactivos, que podrían, por un lado, incrementar el valor biológico de estos pseudocereales y por otro, servir de base para el desarrollo de nuevos ingredientes funcionales.

En esta discusión integradora se presentan los resultados obtenidos en las publicaciones I, II, III y IV, enfocadas a la caracterización de las proteínas de quinoa y a la evaluación de su potencialidad como fuente de péptidos multifuncionales tras la digestión gastrointestinal. A continuación, se discuten los resultados obtenidos en las publicaciones V, VI y VII, en las que se recoge la caracterización, tanto de las proteínas de amaranto como de los péptidos bioactivos liberados a partir de ellas tras su digestión simulando condiciones fisiológicas.

3.1 Caracterización de las proteínas de quinua y evaluación de su potencial como fuente de péptidos multifuncionales tras su digestión gastrointestinal

En la industria alimentaria, el uso de concentrados proteicos obtenidos a partir de nuevas fuentes vegetales está aumentando notablemente debido a diferentes factores intrínsecos a estos concentrados, como las propiedades funcionales de las proteínas constituyentes, su potencialidad como fuente de compuestos bioactivos y el menor contenido de factores anti-nutricionales (Vega-Gálvez y col., 2010). El método más utilizado para obtener dichos concentrados proteicos es la solubilización de las proteínas a pH alcalino (pH 8,0 - pH 12,0) y su posterior precipitación isoeléctrica a pH ácido (pH 4,0 - pH 6,0) (Abugoch y col., 2008). El objetivo de nuestro estudio (Publicación I) fue optimizar el proceso de obtención de concentrados de proteínas de

quinua (CPQ), evaluando el efecto del pH de precipitación sobre el rendimiento y el contenido proteico del CPQ obtenido. En primer lugar, se obtuvo una harina de quinua desengrasada, cuyo contenido en proteína fue del 13,0%, similar al obtenido por otros autores (Karyotis y col., 2003; Abugoch, 2009; Vega-Gálvez y col., 2010). El contenido en proteína de los diferentes QPCs extraídos fue superior al 60%, lográndose el mayor valor (84,3%) cuando la precipitación se produjo a pH 2,0. Sin embargo, el mayor rendimiento de proceso se logró a pH 4,0 de precipitación. También se han obtenido concentrados y aislados proteicos de otras especies vegetales. Así, Mao y Hua, (2012) obtuvieron una harina desengrasada a partir de nuez (*Juglans regia* L.) con un valor de 50,5% de proteína, que sirvió de punto de partida para la obtención un concentrado proteico y un aislado proteico mediante precipitación isoeléctrica a pH 4,5, cuyos porcentajes de proteína fueron del 75,6% y 90,5%, respectivamente. Achouri y col. (2012) extrajeron aislados proteicos a partir de una harina de sésamo (59,0% de proteína) empleando como solvente en la precipitación NaCl a diferentes concentraciones, y obteniendo porcentajes de proteína comprendidos entre el 93,2 y el 100%.

El análisis de las proteínas en los diferentes CPQs se llevó a cabo mediante electroforesis en gel de poliacrilamida nativa (PAGE-nativa) y con agentes desnaturalizantes (PAGE-SDS). Tras el análisis mediante PAGE-nativa pudo observarse similitud en los perfiles de los distintos concentrados pero con una mayor intensidad de las bandas en aquellos CPQs obtenidos tras precipitación a pHs 4,0, 5,0 y 6,0. Sin embargo, el análisis por PAGE-SDS en condiciones no reductoras no permitió ver diferencias en la intensidad de las bandas de los diferentes CPQs. Los resultados obtenidos mediante PAGE-SDS en condiciones reductoras fueron similares a los reportados previamente en la literatura, con bandas más intensas de peso molecular entre 20 y 60 kDa (Brinegar y Goundan, 1993). Las bandas de alto peso molecular (> 130 kDa) no pudieron visualizarse con este análisis. La semilla de quinua está compuesta principalmente por globulinas y albúminas, que representan el 37% y 35% del total de proteínas, respectivamente. Sin embargo, las prolaminas se encuentran en un porcentaje bajo (0,5-7,0%) (Koziol, 1992). Las globulinas se clasifican

en dos grupos dependiendo de su coeficiente de sedimentación: 11S-12S y 7S-8S, de las cuales predominan las proteínas de almacenamiento 11S y 7S. En la quinua, la globulina 11S ha recibido el nombre de chenopodina (Brinegar y Goundan, 1993; Repo-Carrasco y col. 2003). Esta proteína se caracteriza por tener dos conjuntos heterogéneos de polipéptidos, con un tamaño variable entre 30 y 40 kDa (subunidad ácida o AS), y entre 20 y 25 kDa (subunidad básica o AB), las mismas que están unidas por enlaces disulfuro en la proteína nativa. En el CPQ obtenido a pH 4,0, ambas subunidades AS y AB fueron visibles para la chenopodina, tal como se había descrito previamente (Brinegar y col., 1996). Además, dos grades bandas (45-60 kDa) y otras bandas de peso molecular menor a 15 kDa fueron visibles para el QPC. Según Abugoch y col. (2008), la banda de proteína de quinua de 60 kDa podría corresponder a la globulina 7S, mientras que la banda observada al final del gel podría corresponder a la albúmina 2S, que ha sido caracterizada como una proteína de bajo peso molecular (6-8 kDa) (Brinegar y Goundan, 1993; Brinegar y col., 1996).

Dada la importancia de la utilización de los CPQs y debido a la ausencia de datos en la literatura sobre péptidos bioactivos derivados de las proteínas de quinua, se planteó evaluar el potencial de dichas proteínas como fuente de péptidos con capacidad para actuar como antioxidantes, anti-diabéticos y citotóxicos frente a células de cáncer de colon. Por ello se evaluaron la actividad inhibidora las enzimas DPP-IV, α-amilasa y α-glucosidasa, la actividad antioxidante mediada por neutralización de radicales peroxilo y la actividad inhibitoria de la viabilidad de células de cáncer de colon, antes y después de la simulación de la digestión gastrointestinal (Publicación II). El progreso de la digestión y su efecto sobre las proteínas fue estudiado mediante PAGE-SDS, observándose que durante la fase gástrica, la acción de la pepsina provocó la degradación parcial de las proteínas de quinua, resultando en una disminución de la intensidad de las bandas, mientras que la acción de la pancreatina supuso la degradación completa de las proteínas. En el gel sólo pudieron visualizarse las bandas correspondientes a las enzimas utilizadas en la digestión gastroduodenal. Los péptidos con bajo peso molecular liberados tras la hidrólisis de las proteínas no pudieron visualizarse debidos al tipo de gel y las condiciones del análisis.

La DMT2 es un trastorno metabólico crónico considerado como uno de los principales problemas de salud debido a su creciente incidencia y tasa de prevalencia (WHO, 2016). En los últimos años, una de las terapias empleadas para el control de esta enfermedad se basa en la inhibición de varias enzimas implicadas en procesos metabólicos. Por un lado, la inhibición de la DPP-IV ocasiona un mayor contenido de las hormonas incretinas GLP-1 y GIP, que son secretadas en respuesta a la presencia de nutrientes en el lumen intestinal, y actúan estimulando la secreción de insulina por parte de las células β-pancreáticas (Holst y Deacon, 2004; Mentlein, 2005). Además, inhibir la acción de las enzimas responsables de la hidrólisis de los carbohidratos, α-amilasa y α-glucosidasa, se presenta como otra alternativa en el control de la homeostasis de la glucosa en pacientes diabéticos (Johnson y col., 2011). En los últimos años, se han identificado varios péptidos procedentes de proteínas alimentarias con actividad anti-diabética modulada a través de diversos mecanismos (Sanjukta y Rai, 2016; Mojica et al., 2017; Siow et al., 2017).

El CPQ no presentó actividad inhibidora frente a ninguna de las tres enzimas a la máxima concentración ensayada. Sin embargo, las proteínas presentes en el concentrado sirvieron como base para la liberación de péptidos con potencial anti-diabético *in vitro*. El digerido obtenido al final de la fase gástrica mostró efectos moderados como inhibidor frente a la DPP-IV, aunque no mostró habilidad para inhibir la α-amilasa y α-glucosidasa. Este comportamiento también ha sido descrito para digeridos obtenidos a partir de otras fuentes proteicas. Así, por ejemplo, digeridos de α-lactoalbúmina y la β-lactoglobulina con pepsina fueron caracterizados por su actividad inhibidora de la DPP-IV, con valores de IC_{50} que oscilaron entre 0,04 mg de proteína/mL y 1,28 mg de proteína/mL, sin mostrar ningún efecto inhibidor frente a la α-glucosidasa (Lacroix y Li-Chan, 2013). En nuestro estudio, la acción continuada en el tiempo de la pancreatina permitió la liberación de péptidos con una potente actividad inhibidora de la DPP-IV y α-amilasa y una moderada acción frente a la α-glucosidasa. El valor de IC_{50} del digerido al final del proceso digestivo relativo a la inhibición de la DPP-IV (0,25 ± 0,01 mg proteína/mL) fue inferior al determinado para los péptidos liberados durante la digestión gastrointestinal *in vitro* de la glutelina de amaranto

(Velarde-Salcedo y col., 2013) aunque superior al calculado para digeridos con pepsina y una mezcla de tripsina-pancreatina de proteínas de cereales (avena y alforfón) (Wang y col., 2015). En el caso de la inhibición de la α-amilasa, fue notable el incremento de la actividad durante la fase duodenal alcanzándose un valor de IC_{50} de 0,19 ± 0,02 mg proteína/mL al final del proceso digestivo. Hasta la fecha, se dispone de pocos datos de los efectos inhibidores sobre esta enzima de hidrolizados de proteínas alimentarias. Mojica y de Mejía (2016) obtuvieron una inhibición del 53,4% de las proteínas de judía negra hidrolizadas con Alcalasa® a una concentración final de 0,33 mg de hidrolizado/mL. En el caso de la actividad inhibidora de la α-glucosidasa, los primeros 60 min de incubación con la pancreatina fueron suficientes para liberar péptidos con moderada actividad manteniéndose intactos tras la acción más prolongada en el tiempo de la pancreatina. La actividad inhibitoria de α-glucosidasa fue más potente que la determinada en un hidrolizado del músculo de sardina con proteasas alcalinas (IC_{50} = 48,7 mg/mL, Matsui y col., 1996), y en los hidrolizados de las proteínas de las semillas de cáñamo con Alcalasa®, que alcanzaron un 50% de inhibición enzimática a una concentración de 5 mg/mL (Ren y col., 2016). En ambos ensayos, los autores utilizaron α-glucosidasa microbiana, a diferencia de la α-glucosidasa intestinal de rata empleada en nuestro estudio. Las variaciones estructurales entre las enzimas microbiana y mamífera podrían ser las responsables de la unión diferente de los inhibidores a su sitio activo, y por tanto, de los diferentes niveles de actividad inhibidora observada. Algunos inhibidores sintéticos han demostrado ejercer una potente actividad sobre la α-glucosidasa de mamíferos, sin afectar a la enzima procedente de la levadura de panadería (Oki y col., 1999). En estudios que evalúan la actividad inhibidora de péptidos derivados de los alimentos, se prefiere la α-glucosidasa intestinal de mamíferos debido a la mayor relevancia para la salud humana. Además, también se prefiere la maltosa como sustrato para esta enzima, ya que es el principal producto digestivo del almidón en el intestino delgado (Lacroix y Li-Chan, 2013).

El estrés oxidativo, provocado por un desequilibrio entre las especies reactivas resultantes del metabolismo celular y las defensas del organismo, ha sido asociado a múltiples desórdenes crónicos, como la diabetes, anteriormente citada, los trastornos

neurológicos y cardiovasculares, el cáncer y el propio envejecimiento (Fiaschi y Chiarugi, 2012). En los últimos años, se ha incrementado el interés por compuestos naturales con capacidad para prevenir o retrasar estos desórdenes, actuando mediante diversos mecanismos, entre ellos como protectores del estrés oxidativo. Por lo tanto, se ha intensificado la búsqueda de antioxidantes naturales eficaces y sin los efectos secundarios asociados a los antioxidantes sintéticos, como el BHA y el BHT (Chen y col., 1992). Entre estos antioxidantes, los péptidos procedentes de fuentes alimentarias y principalmente, fuentes vegetales, se han posicionado como una alternativa prometedora en la prevención de enfermedades cardiovasculares, inflamación y cáncer (Cavazos y Gonzalez de Mejia, 2013). Por lo tanto, resulta de interés investigar esta actividad antioxidante en péptidos de otras fuentes, como es el caso de la quinoa. En nuestro estudio, la acción de proteasas endógenas pudo permitir la liberación de aminoácidos y/o péptidos con una ligera actividad neutralizante de radicales peroxilo, como había sido descrito previamente (Nongonierma y col. 2015). Sin embargo, fueron las enzimas digestivas las principales responsables de degradar las proteínas de quinoa y liberar péptidos con una potente actividad antioxidante, principalmente durante la fase duodenal. Los péptidos liberados durante los primeros minutos de incubación con la pancreatina resistieron una acción más prolongada de esta enzima, pudiendo ejercer un efecto antioxidante a nivel local relevante. Los valores de ORAC obtenidos en este estudio fueron superiores a los determinados por Nongonierma y col., (2015) para la proteína de quinua hidrolizada con papaína (0,50 μmol Trolox equivalente/mg de proteína), lo que sugiere el papel importante de la digestión gastrointestinal en la liberación de péptidos antioxidantes a partir de esta proteína vegetal. Estudios anteriores también habían descrito el impacto de la digestión gastrointestinal en la liberación de péptidos bioactivos a partir de las proteínas precursoras, así como en la modificación o descomposición de péptidos con propiedades antioxidantes (Gallegos y col., 2015; Gallego y col., 2017). Además, la actividad de los péptidos procedentes de la quinoa fueron comparables a la determinada para péptidos de origen animal, como la α-lactoalbúmina y la β-lactoglobulina (Hernández y col., 2005).

Dado que existe una interacción continua y directa entre el tracto digestivo y los alimentos, los compuestos dietéticos representan una interesante fuente de agentes quimiopreventivos para la salud gastrointestinal (Moura y col., 2015). Algunos componentes de los alimentos poseen la capacidad de proteger el cuerpo de la proliferación celular maligna causada por el estrés oxidativo inducido por los radicales libres. Entre ellos, los péptidos bioactivos derivados de los alimentos están atrayendo la atención debido a su actividad demostrada sobre la promoción de la salud, sin causar efectos secundarios adversos (Chakrabarti y col., 2014; Hernández-Ledesma y Hsieh, 2017). Sin embargo, el número de estudios enfocados en evaluar la actividad citotóxica de péptidos alimentarios es todavía limitado y se han llevado a cabo principalmente con hidrolizados de proteínas animales. Por esta razón, evaluar el potencial de la proteína de quinua como fuente de péptidos inhibidores de la viabilidad celular resultaba de gran interés. De forma preliminar, usando células Caco-2, pudo comprobarse que durante el proceso digestivo se liberaron péptidos con efectos citotóxicos. Esta actividad fue notable y dosis-dependiente en el digerido gástrico, alcanzándose el máximo de inhibición (81,2%) a la concentración de 4,0 mg de proteína/mL. El valor de IC_{50} de dicho digerido fue de 0,843 ± 0,001 mg de proteína/mL. Esta actividad fue superior a la mostrada por digeridos de proteínas animales, como los resultantes de la acción de la pepsina sobre las huevas de *Labeo rohita* (rohu), que inhibieron la viabilidad de las células Caco-2 en un 65,0 ± 13,9% a la concentración de 9 mg/mL (Chalamaiah y col., 2015). Igualmente, menores efectos citotóxicos fueron mostrados por los hidrolizados del pez locha con papaína, con un máximo de reducción de la proliferación de células Caco-2 a 20 mg/mL (You, y col., 2011).

Cabe destacar que la actividad inhibidora de la viabilidad celular de los digeridos gastroduodenales aumentó significativamente en comparación con la demostrada por el digerido gástrico, con valores de IC_{50} para los digeridos a 60 y 120 minutos de incubación con la pancreatina de 0,191 ± 0.003 mg proteína/mL y 0,180 ± 0,001 mg proteína/mL, respectivamente. Este resultado indica que los péptidos más potentes que inhiben la viabilidad de las células Caco-2 fueron liberados por acción de la enzima pancreática, aunque no se puede descartar la contribución de esta enzima y

las sales biliares como agentes citotóxicos, ya que el valor de IC_{50} determinado para los blancos de digestión duodenal fueron 1,241 ± 0,040 mg/mL. Un estudio más pormenorizado fue llevado a cabo en el digerido obtenido a los 60 minutos de fase duodenal, empleando otros dos modelos celulares de cáncer de colon (HT-29 y HCT-116). Aunque los efectos citotóxicos mostrados para las tres líneas celulares fueron similares, es de destacar la mayor sensibilidad de las HT-29 y HCT-116 a la enzima y las sales biliares, y por lo tanto, una mayor susceptibilidad de las células Caco-2 a los péptidos derivados de la proteína de quinua. Recientemente, se ha descrito alta sensibilidad de las células Caco-2 en comparación con las células HT-29 y HCT-116 para el digerido gastroduodenal obtenido a partir de proteínas de soja germinadas (González-Montoya y col., 2017). Las diferencias en la actividad citotóxica entre las líneas celulares pueden deberse a diferencias en la composición de la membrana celular, fluidez y área de superficie de cada una de las líneas celulares (Leuschner y Hansel, 2004).

Una vez comprobada la multifuncionalidad de los péptidos liberados a partir de las proteínas de quinua durante el proceso digestivo y con el objetivo de estudiar la influencia del peso molecular en las diferentes actividades, los digeridos intestinales, que fueron lo que habían mostrado mayor actividad y podrían contener péptidos potencialmente biodisponibles, fueron sometidos a ultrafiltración para separar las fracciones peptídicas mayor y menor a 5 kDa. De forma general, pudo observarse que los péptidos de pequeño tamaño fueron los principales responsables de la actividad anti-diabética *in vitro* y la actividad antioxidante, mientras que los péptidos de tamaño mayor a 5 kDa contribuyeron en mayor medida sobre la actividad citotóxica. En el caso de la actividad inhibidora de la DPP-IV, los menores valores de IC_{50} determinados en las fracciones que contenían los péptidos más cortos concuerdan con estudios previos que habían asociado esta actividad con péptidos de bajo peso molecular (Power y col., 2014). Sin embargo, la mayor actividad mostrada por el digerido completo con respecto a la fracción de pequeño tamaño sugiere o bien, que el proceso de ultrafiltración permitió el paso de péptidos bioactivos pequeños a la fracción mayor a 5 kDa, contribuyendo en la actividad mostrada por dicha fracción o bien, que péptidos de

mayor peso molecular podrían haber contribuido en la actividad mostrada por el digerido sin ultrafiltrar. Así, Velarde-Salcedo y col., (2013) identificaron péptidos de 13 aminoácidos procedentes de la glutelina de amaranto con capacidad para interactuar con el sitio de dimerización de la enzima DPP-IV inhibiendo la formación del dímero activo. De forma similar, una fracción que contenía péptidos derivados de las proteínas de suero con peso molecular entre 3 y 10 kDa demostraron una actividad inhibidora de la DPP-IV mayor que la ejercida por la fracción de menor peso molecular (Babij y col., 2014). Además, tres péptidos, con una longitud de entre 13 y 15 aminoácidos, fueron identificados en hidrolizados de proteína de atún y caracterizados como inhibidores de la DPP-IV (Huang y col., 2012). Estos resultados hicieron que los autores sugirieran la influencia que podrían ejercer sobre dicha actividad otros factores inherentes a los péptidos como su composición y secuencia de aminoácidos, independientemente de su longitud. Lacroix y Li-Chan (2012) también indicaron que la aparente actividad inhibitoria de la DPP-IV de péptidos con alto peso molecular podría atribuirse a sus características estructurales, actuando como un sustrato e interfiriendo en la unión de la enzima al sustrato empleado en el método de análisis. Los péptidos contenidos en la fracción $<$ 5 kDa también mostraron una potente actividad inhibidora de la α-amilasa y una moderada actividad frente a la α-glucosidasa, indicando que el menor tamaño de los fragmentos peptídicos favorece la inhibición de las enzimas implicadas en la hidrólisis de los carbohidratos. Estudios previos han demostrado que péptidos de peso molecular inferior a 3 kDa derivados de las glutelinas del salvado de arroz (Uraipong y Zhao, 2016) o de las proteínas de la judía común (Oseguera-Toledo y col., 2015) son más potentes inhibiendo la α-glucosidasa que péptidos de mayor tamaño.

Al igual que para la actividad anti-diabética *in vitro*, la capacidad neutralizante de radicales peroxilo también pudo ser asociada a los péptidos de pequeño tamaño. Esta asociación entre la capacidad neutralizante de radicales libres y el menor peso molecular de los péptidos también ha sido descrita por otros autores (Roblet y col., 2012; Dong y col., 2013). Aluko y Monu (2003) estudiaron la capacidad neutralizante de radicales DPPH de los péptidos procedentes de la hidrólisis de proteínas de quinua con Alcalasa®, observando una mayor capacidad para la fracción < 5 kDa en relación a la

fracción < 10 kDa y el hidrolizado de partida, que no presentó ningún efecto antioxidante. Sin embargo, en nuestro estudio la contribución a la actividad de péptidos de mayor tamaño no puede ser descartada. Se ha descrito que, adicionalmente a su tamaño, otros factores de los péptidos, como su composición aminoacídica, su estructura y su carácter hidrofóbico ejercen una influencia sobre el mecanismo y la eficiencia como agentes antioxidantes (Gallego y col., 2017).

A diferencia de las anteriores actividades, los efectos citotóxicos de los digeridos gastroduodenales del CPQ pudieron ser atribuidos mayoritariamente a los péptidos contenidos en la fracción > 5 kDa. Los valores de IC_{50} para esta fracción obtenida a partir del digerido a los 60 minutos de incubación con pancreatina fueron 0,239 ± 0,001 mg proteína/mL para células Caco-2, 0,085 ± 0,003 mg proteína/mL para células HT-29 y 0,176 ± 0,000 mg de proteína/mL para células HCT-116. Las enzimas digestivas retenidas en la fracción >5 kDa y las sales biliares que pasaron a través del filtro de membrana apareciendo en la fracción <5 kDa pudieron ser responsables de los efectos citotóxicos mostrados por las fracciones recogidas a partir de los blancos de digestión. La asociación entre el peso molecular de los péptidos y su actividad citotóxica también había sido estudiada por Rayaprolu y col., (2013) quienes describieron que la fracción de peso molecular entre 10 y 50 kDa obtenida de la proteína de harina de soja fue activa inhibiendo la viabilidad de células cancerosas de colon, hígado y pulmón. González-Montoya y col., (2017) también describieron alta actividad para las fracciones de alto peso molecular obtenidas a partir de digeridos de proteínas de soja germinada. No obstante, Kannan y col., (2008) encontraron que la fracción < 5 kDa de péptidos liberados tras la hidrólisis de proteínas de salvado de arroz tenía una mayor actividad anti-proliferativa frente a células HepG-2 y Caco-2 que las fracciones con peso molecular entre 5 y 10 kDa y mayor a 10 kDa. Aunque se ha considerado que la mayor movilidad molecular y la difusión de los péptidos de bajo peso molecular mejoran las interacciones con los componentes de las células cancerosas y potencian la actividad antiproliferativa (Jumeri y Kim, 2011), en nuestro estudio, otros aspectos como la composición de aminoácidos y la hidrofobicidad del péptido podrían tener un impacto positivo en la actividad de la fracción > 5 kDa.

Con el objetivo de identificar los péptidos potencialmente responsables de la multifuncionalidad de los digeridos gastroduodenales, la muestra obtenida a los 60 minutos de incubación con la pancreatina fue sometida a HPLC a escala preparativa, recogiendo tres fracciones peptídicas (F-1, F-2 y F-3). La concentración de péptidos de F-1, F-2 y F-3 fue de 38,0%, 50,0% y 48,0%, respectivamente. El contenido peptídico inferior de F-1 podría ser debido a la elución de las sales hidrófilas en esta fracción al comienzo del gradiente de separación. La composición de aminoácidos se muestra en la Publicación III. Las condiciones de análisis no permitieron la identificación de triptófano debido a su destrucción por la hidrólisis ácida. El contenido total de aminoácidos del digerido fue del 49,2%, con presencia tanto de aminoácidos esenciales como no-esenciales. El porcentaje de relación entre los aminoácidos esenciales y los totales fue del 31%, que se considera como el porcentaje necesario para que una proteína ideal cumpla con los requerimientos de aminoácidos en un individuo. Los aminoácidos cargados negativamente (15,0%) fueron los más abundantes, seguidos de los aminoácidos hidrofóbicos (14,8%), los cargados positivamente (8,6%), de cadena ramificada (7,2%) y los aminoácidos aromáticos (4,1%). El contenido total de aminoácidos de la fracción F-1 (67,9%) y F-2 (86,6%) fue 1,4 y 1,8 veces superior al determinado en el digerido completo. Los niveles de aminoácidos aromáticos fenilalanina y tirosina aumentaron con el tiempo de retención hasta 1,1 y 3,6 veces en F-1 y F-2, respectivamente. También se observaron incrementos en los niveles de aminoácidos de cadena ramificada e hidrofóbicos en estas dos fracciones en comparación con DI60. En contraste, el contenido de aminoácidos cargados positivamente en la fracción F-2 fue 1,8 veces menor que el DI60. Estos resultados apoyan el hecho de que el tiempo de resistencia en la columna está influenciado principalmente por la hidrofobicidad del péptido (Girgih y col., 2013). Sin embargo, las disminuciones observadas en los niveles de aminoácidos hidrofóbicos y aromáticos en la fracción F-3 sugieren que otros factores como el tamaño del péptido deben tenerse en cuenta durante su elución en HPLC. Así, el análisis de la distribución de masas de péptidos mostró que los péptidos con un peso molecular inferior a 1100 kDa eran los más abundantes en la fracción F-1 (30% de los aminoácidos totales),

disminuyendo este porcentaje a 23,0 y 16,5% en las fracciones F-2 y F-3, respectivamente. La fracción F-2 presentó el contenido más elevado de péptidos con peso molecular superior a 1101 kDa (15%). Aunque se esperaba que los péptidos más largos estuvieran presentes principalmente en la fracción F-3, debido a las limitaciones del método no fue posible confirmar esta hipótesis.

Las dos primeras fracciones presentaron efectos inhibidores sobre las tres enzimas implicadas en reacciones metabólicas, mientras que F-3 sólo mostró actividad inhibidora de la DPP-IV y α-glucosidasa. La fracción F-2 fue la que mostró los efectos más potentes, siendo los péptidos contenidos en ella los principales contribuyentes en la actividad mostrada por el digerido sin fraccionar. Sin embargo, los péptidos contenidos en las otras dos fracciones también pudieron contribuir, aunque en menor medida, en la actividad inhibidora de la DPP-IV.

Los valores más elevados de actividad antioxidante se determinaron en las fracciones F-1 y F-2, resultado que concuerda con la asociación de la capacidad de neutralización de radicales con hidrolizados o péptidos con un elevado contenido en aminoácidos hidrofóbicos y/o aromáticos (Rajapakse y col., 2005). Los aminoácidos hidrofóbicos representaron el 21,7 y el 38,9% de los aminoácidos totales en la fracción F-1 y F-2, respectivamente. Esto podría aumentar la solubilidad de los péptidos en los lípidos, facilitando una mejor interacción con los radicales libres. Además, los aminoácidos aromáticos podrían donar protones a radicales deficientes de electrones, manteniendo su estabilidad. Su contenido en F-1 y F-2 también fue importante, 4,6% y 15,1%, respectivamente. Por lo tanto, la composición de aminoácidos de las fracciones recogidas a partir del digerido DI60 fue compatible con la presencia de péptidos con capacidad antioxidante.

Mientras que las fracciones F-1 y F-2 sólo afectaron a la viabilidad de las células HT-29 y HCT-116, la fracción F-3 ejerció potentes efectos citotóxicos frente a las tres líneas celulares de cáncer. Los valores de IC_{50} calculados frente a las células Caco-2, HT-29 y HCT-116 fueron 0,256 ± 0,004, 0,195 ± 0,001 y 0,193 ± 0,003 mg péptido/mL, respectivamente. Estos valores fueron similares o ligeramente inferiores a

los mostrados por el digerido completo, lo que sugiere que los péptidos contenidos en F-3 fueron los principales responsables de los efectos anti-proliferativos a nivel intestinal mostrado por los digeridos de proteínas de quinua. Dichos efectos no se pueden atribuir totalmente a los péptidos que contienen aminoácidos aromáticos y/o hidrofóbicos, ya que los contenidos de estos residuos fueron menores en la F-3 en comparación con las otras dos fracciones. Como se ha mencionado anteriormente, la actividad anti-proliferativa también podría estar relacionada con la presencia de péptidos específicos que ejercen una citotoxicidad directa sobre las células cancerosas. Así por ejemplo, se ha demostrado que el péptido EGRPR, extraído del salvado de arroz, causa la inhibición del 85,0% del crecimiento de células de cáncer de colon Caco-2 y HCT-116 a una dosis de 600-700 µg/mL (Kannan y col., 2010). En el caso de la quinua, la presencia del péptido quimiopreventivo lunasina recientemente descrita por Ren y col., (2017) podría explicar parcialmente los potentes efectos anticancerígenos observados en los digeridos, aunque sería necesario confirmar esta hipótesis identificando este péptido o alguno de los péptidos liberados tras su digestión en las fracciones cromatográficas recogidas en nuestro estudio.

Debido a que cada una de las fracciones caracterizadas mostraba efectos biológicos, se planteó su análisis por HPLC-MS/MS para identificar los péptidos potencialmente responsables de dichos efectos. Se pudieron detectar un gran número de fragmentos mediante este análisis. Sin embargo, debido a que la mayoría de las proteínas de quinoa no están secuenciadas, sólo fue posible identificar 17 péptidos, cuatro presentes en F-1, cinco presentes en F-2 y ocho en F-3. De estos péptidos, 13 procedieron de la globulina 11S o de sus variantes A y B, dos de la maturasa K, uno de la betaína aldehído deshidrogenasa y uno de la sintasa de almidón.

En base a su secuencia y a los estudios existentes sobre la relación estructura/actividad, tres de los péptidos identificados en la fracción F-2 fueron sintetizados y evaluados con repecto a su actividad inhibidora de la DPP-IV, α-amilasa y α-glucosidasa. El péptido DKDYPK no presentó actividad frente a la DPP-IV, mientras que los valores de IC_{20} calculados para los fragmentos GEHGSDGNV e IQAEGGLT fueron 1061 µM y 267,81 µM, respectivamente. Ninguno de estos péptidos contenía

prolina en la primera, segunda, tercera o cuarta posición amino-terminal, característica que ha sido definida como determinante en la actividad inhibitoria de la DPP-IV (Pieter, 2006). Los mayores efectos inhibitorios mostrados por el péptido IQAEGGLT pudieron deberse a la presencia de isoleucina como aminoácido amino-terminal. Recientes estudios *in silico* han demostrado que potentes péptidos inhibidores de la DPP-IV generalmente contienen un aminoácido de cadena ramificada (leucina, isoleucina, o valina), o un residuo aromático con un grupo polar en la cadena lateral (principalmente triptófano) en su posición N-terminal (Nongonierma y FitzGerald, 2014; Tulipano y col., 2015). La mezcla equimolecular de los tres péptidos sintetizados presentó una mayor actividad inhibidora de la DPP-IV que los péptidos analizados individualmente, calculándose un valor de IC_{30} de 228,03 μM. Este resultado indica que las posibles interacciones entre los péptidos favorecieron la inhibición de la enzima. Por lo tanto, estos péptidos en combinación con aquellos que no han sido identificados podrían ser determinantes sobre las propiedades inhibidoras de la DPP-IV mostradas por la fracción F-2.

El mecanismo de acción propuesto para la actividad inhibidora de péptidos sobre la α-amilasa está relacionado con su capacidad para formar un enlace de hidrógeno con los residuos del sitio de unión del sustrato a la enzima, inhibiendo dicha unión (Siow y Gan, 2016; Siow y Gan 2017). La α-amilasa tiene una serie de residuos aromáticos que se encuentran en el sitio de unión al sustrato que podrían interactuar directamente con el sustrato. Se ha encontrado que estos residuos están probablemente unidos a los péptidos, especialmente por sus residuos aromáticos (fenilalanina, triptófano, y tirosina). Por tanto, las interacciones entre los residuos aromáticos de la enzima y del péptido a través de enlaces de hidrógeno, interacciones electrostáticas y de Van der Waals, parecen estar implicados críticamente en la acción inhibidora de los péptidos sobre la α-amilasa (Siow y col., 2017). El péptido IQAEGGLT no contiene ningún residuo aromático, y sólo una tirosina está presente en el péptido DKDYPK, hecho que podría explicar la falta de la actividad inhibitoria mostrada por estas dos secuencias. El péptido GEHGSDGNV mostró una ligera actividad inhibidora de la α-amilasa con un valor de IC_{20} de 921,48 μM. Esta actividad podría deberse a la

presencia de la glicina, ya que se ha descrito la influencia positiva ejercida por este aminoácido sobre las propiedades inhibidoras de los péptidos (Ngoh y Gan, 2016), aunque no puede descartarse también la contribución potencial de otros residuos de aminoácidos cuya implicación todavía no ha sido estudiada.

En el caso de la α-glucosidasa, Roskar y col. (2015) sugirieron que compuestos insulino-miméticos son mejores inhibidores que otros compuestos de naturaleza fenólica, incluidos flavonoides y taninos, cuya actividad inhibidora sobre esta enzima ha sido descrita (Gonçalves y col., 2011; You y col., 2012). Además, estudios realizados en la última década han demostrado el gran potencial inhibidor de los péptidos derivados de los alimentos, debido a su afinidad y especificidad sobre la α-glucosidasa (Lacroix y Li-Chan, 2013; Ren y col., 2016; Yu y col., 2011). El mecanismo exacto por el cual los péptidos podrían inhibir la α-glucosidasa se desconoce, pero se ha sugerido que pueden ejercer su actividad inhibidora uniéndose al sitio activo de la enzima a través de interacciones hidrofóbicas, tal como se ha reportado para otros inhibidores (Bharathan y col., 2008). Ren y col. (2016) confirmaron el papel de los aminoácidos hidrofóbicos en los péptidos con propiedades inhibitorias de la α-glucosidasa. El péptido IQAEGGLT, que contiene tres residuos hidrofóbicos, presentó una actividad inhibitoria potente sobre la α-glucosidasa, con un valor de IC_{50} de 109,48 μM. Sin embargo, las secuencias DKDYPK y GEHGSDGNV presentaron porcentajes bajos de inhibición a la concentración de 250 μM, que pudo ser debido al mayor carácter hidrofílico de estos péptidos.

Los aminoácidos triptófano, tirosina y metionina se han descrito como los principales contribuyentes en la capacidad neutralizante de radicales peroxilo de los péptidos, con un ligero papel para los residuos de cisteína, histidina y fenilalanina (Hernández-Ledesma y col., 2005). Por lo tanto, los péptidos derivados de la quinua LWREGM (F-1), DKDYPK (F-2) y DVYSPEAG, IFQEYI, Y RELGEWGI (F-3), podrían ser responsables de los efectos antioxidantes observados en cada una de estas fracciones.

En resumen, nuestros resultados demuestran el potencial de las proteínas de quinua como fuente de péptidos bioactivos, que podrían usarse en el desarrollo de alimentos funcionales o nutracéuticos para la prevención y/o manejo de la DMT2 y la reducción de enfermedades asociadas al estrés oxidativo, incluido el cáncer. Sin embargo, los métodos *in vitro* presentan limitaciones, ya que ninguno de estos métodos proporciona resultados inequívocos, existiendo además una falta de estandarización en los criterios de valoración (Carocho y Ferreira, 2013).

En este sentido, en los últimos años se viene empleando modelos alternativos que permitan evaluar la biodisponibilidad, el metabolismo y la bioactividad de los compuestos alimenticios como una herramienta previa a los modelos animales superiores y ensayos clínicos que suponen mayor complejidad, coste e implicaciones éticas (Power y col., 2013; Wan y col., 2015). El pez cebra (*Danio rerio*) está considerado como un modelo emergente de vertebrado utilizado para estudiar eventos toxicológicos, como las alteraciones neuroquímicas promovidas por metales pesados, así como para evaluar nuevos medicamentos y productos químicos (Senger y col., 2006). Recientemente, este modelo se ha usado para evaluar la actividad antioxidante de compuestos de origen alimentario (Carrillo y col., 2016).

Por lo tanto, este modelo resulta interesante para evaluar la actividad inhibidora de la peroxidación lipídica de péptidos procedentes de la quinua (Publicación IV). Previamente a la evaluación de la actividad antioxidante de los digeridos fue necesario descartar efectos citotóxicos de dichos digeridos sobre el pez cebra. Tras el tratamiento con los digeridos a diferentes concentraciones, las larvas presentaron una morfo-fisiología similar a las larvas control (sin muestra), lo que confirma la ausencia de efectos citotóxicos en este modelo. Se comprobó que la mayor hidrólisis de las proteínas de quinua resultantes de la incubación con pepsina a su pH óptimo favoreció la capacidad inhibidora de la peroxidación, alcanzando un porcentaje de inhibición del 75,15%. Durante la incubación con pancreatina se liberaron nuevos péptidos más potentes como antioxidantes y así, el hidrolizado obtenido al final del proceso digestivo presentó un porcentaje de inhibición de la peroxidación lipídica del 82,10%, similar al porcentaje obtenido para el control positivo (BHT) utilizado en el ensayo (87,13% de

inhibición lipídica a una concentración de 0,1 mg/mL). Estudios previos han descrito la capacidad de los péptidos derivados de diferentes fuentes alimentarias para inhibir la peroxidación lipídica. Por ejemplo, Chen y col. (1998) describieron esta capacidad para hidrolizados de proteínas marinas. Aunque el mecanismo exacto de los péptidos para actuar como antioxidantes no está claramente conocido, se sabe que el tamaño, la presencia de histidina y de algunos aminoácidos aromáticos desempeñan un papel importante (Carrillo y col., 2016; Qian y col., 2008). Se ha sugerido que la presencia de los aminoácidos fenilalanina y leucina es también importante para ejercer los efectos antioxidantes. Las proteínas de quinua son conocidas por ser ricas en los aminoácidos leucina, histidina, fenilalanina y arginina, por lo que los péptidos que contienen estos aminoácidos podrían ser liberados durante los procesos digestivos y ejercer actividad antioxidante a través de la inhibición de la peroxidación de lípidos (Abugoch, 2009).

3.2 Proteínas de amaranto (*Amaranthus caudatus*): caracterización y potencialidad como fuente de péptidos multifuncionales tras su digestión gastrointestinal *in vitro*

Con el objetivo de optimizar las condiciones de extracción de las proteínas a partir del amaranto, se obtuvieron diferentes concentrados (CPA) evaluando el efecto de los pHs de solubilización y precipitación, empleando agua o NaCl como disolventes (Publicación V). Previamente, se obtuvo una harina de amaranto desengrasada que presentó un contenido en proteína del 13,9%, similar al descrito para *Amaranthus hypochondriacus* y para varias plantas dicotiledóneas (Marcone y col., 1998; Tandang y col., 2012). Tomando como partida esta harina se obtuvieron diferentes CPAs, determinándose el mayor contenido proteico (50,8%) en aquel concentrado obtenido tras la precipitación a pH 4,0. El contenido en carbohidratos de este CPA fue del 30,5%. El rendimiento del proceso fue superior empleando NaCl como solvente en comparación con el agua, de forma similar a lo descrito por otros autores para la extracción de concentrados a partir de otras fuentes vegetales Achouri y col., (2012, 2013). Sin embargo, la necesidad de un proceso de diálisis posterior a la extracción del concentrado limitaba el uso de la solución salina para la obtención de concentrados a partir del amaranto.

Tras la optimización del proceso se obtuvo un CPA con un contenido en proteína del 53,6%, similar al obtenido por Escudero y col. (2004) usando harina de *A. cruentus* y pH 11,0 para la solubilización de las proteínas. La caracterización del CPA se llevó a cabo mediante PAGE-SDS, observándose bandas de pesos moleculares entre 6,5 y 150 kDa, siendo más intensas aquellas de 78, 55, 30-35, 20-25, 17, 15, 13 y 11 kDa. Este perfil proteico es similar al descrito previamente en los estudios realizados para las proteínas de otras especies de amaranto. De acuerdo con Gamel y col. (2005), las albúminas muestran tres bandas, dos entre 42 y 25 kDa y una por debajo de 20 kDa, mientras que las globulinas muestran bandas de 78 y 54 kDa, dos bandas entre 40 y 38 kDa, tres bandas entre 27 y 21 kDa y dos bandas a 15 y 14 kDa. Las glutelinas presentan dos bandas entre 67 y 43 kDa, y dos bandas entre 38 y 35 kDa, y finalmente las bandas de prolaminas se encuentran entre 34 y 20 kDa.

Estudios previos habían comprobado el papel de las proteínas de otras especies de amaranto como *A. hypochondriacus* como fuente de péptidos anti-inflamatorios e inhibidores de la ECA (Silva y col., 2008; Vecchi y Añón, 2009; Maldonado-Cervantes y col., 2010; Velarde-Salcedo y col., 2013). Sin embargo, los estudios empleando otras especies como *A. cruentus* y enfocados en otras actividades son más limitados (Manolio y col., 2015; Orsini y col., 2016). En el caso de *A. caudatus*, hasta el momento no existe ningún estudio sobre la potencialidad de sus proteínas como fuente de péptidos bioactivos. Por lo tanto, nos planteamos evaluar la multifuncionalidad de digeridos de amaranto tras simular la digestión gastrointestinal e identificar los péptidos potencialmente responsables de los efectos observados en esta especie (Publicación VI). Al igual que se había observado con la quinua, la acción de la pepsina resultó en una degradación parcial de las proteínas de amaranto, disminuyendo la intensidad de las bandas observadas mediante el análisis electroforético, principalmente de aquellas con peso molecular superior a 25 kDa. La degradación completa de las proteínas fue notable tras la incubación con la pancreatina. Del mismo modo, Orsini Delgado y col. (2011) describieron la disminución de la intensidad total de las bandas de proteínas de *A. mantegazzianus* digeridas tras un proceso simulado de digestión gastroduodenal *in vitro.* Los péptidos liberados tras la

degradación de las proteínas no pudieron ser visibles debido a las características del gel y las condiciones del ensayo.

Se evaluaron diferentes actividades biológicas en los digeridos gástricos y gastroduodenales, concretamente la actividad inhibidora de la ECA, la actividad antioxidante, la actividad inhibidora de la DPP-IV, α-amilasa y de la viabilidad de células de cáncer de colon. Al inicio de la digestión, péptidos resultantes de la acción de proteasas endógenas pudieron ser responsables de la actividad inhibidora de la ECA mostrada por la muestra obtenida a tiempo 0 de la digestión. Este resultado concuerda con los obtenidos por Fritz y col. (2011) que observaron un porcentaje de inhibición de la ECA mayor al 10% para el aislado proteico obtenido de *A. mantegazzianus*. Sin embargo, previamente, Tiengo y col. (2009) había descrito la inactividad de las proteínas intactas de amaranto, ya que el valor de IC_{50} determinado para un concentrado proteico extraído de *A. cruentus* fue de 12 mg proteína/mL. La actividad inhibidora de la ECA aumentó notablemente tras la acción de la pepsina, volviendo a los valores iniciales tras la fase duodenal. Este resultado indicó que los péptidos liberados por la acción de la enzima gástrica fueron degradados por la acción de la pancreatina perdiendo parte de su actividad. A pesar de esta pérdida, los valores del IC_{50} obtenidos fueron inferiores al obtenido por Tiengo y col. (2009) (439,0 ± 18 µg proteína/mL) para el concentrado proteico de *A. cruentus* digerido bajo condiciones simuladas. Igualmente, nuestros digeridos fueron más activos que los hidrolizados de las fracciones de globulina y albúmina extraídas de *A. hypochondriacus* e hidrolizadas con Alcalasa® (IC_{50} entre 925 y 1887 µg/mL, Soriano-Santos y Escalona-Buendía, 2015). Los hidrolizados de otras especies vegetales también han mostrado menor actividad inhibidora de la ECA. Así, el IC_{50} de un digerido de proteína de soja fue 0,28 mg/mL (Lo y Li-Chan, 2005) y los de hidrolizados de harina desengrasada de lupino variaron entre 0,21 y 0,33 mg/mL (Yoshie-Stark y col., 2004).

Los péptidos más potentes como neutralizantes de radicales peroxilo se liberaron durante los primeros 60 minutos de incubación con la pancreatina, degradándose por la acción más prolongada en el tiempo de esta enzima. La actividad obtenida en nuestros digeridos gastrointestinales fue superior a la descrita por Orsini

Delgado y col. (2011) para digeridos gastrointestinales de amaranto (0,80-1,16 µmol Trolox equivalente/mg proteína), que puede deberse a la diferente especie empleada y las distintas condiciones del proceso de digestión.

Antes de la digestión, el CPA no presentó actividad anti-diabética *in vitro.* Sin embargo, potentes péptidos inhibidores de la DPP-IV se liberaron tras la acción de la pepsina, aunque estos no mostraron efectos sobre la α-amilasa pancreática. Nuevos péptidos con mayor actividad frente a ambas enzimas metabólicas se formaron tras la acción de la pancreatina. Los valores de actividad inhibidora de la DPP-IV fueron superiores a los descritos por Velarde-Salcedo y col. (2013) para digeridos de amaranto y por Nongonierma y Fitzgerald (2015) para digeridos de proteínas de cáñamo, guisante, arroz y soja, aunque comparables a los determinados para los digeridos de quinua. La potencia inhibidora de la α-amilasa de los digeridos duodenales fue similar a la demostrada para otros sustratos vegetales, como los péptidos aislados de la semilla de comino, los hidrolizados de judía pinta Protamex y los péptidos aislados a partir de estos últimos hidrolizados (Ngoh y Gan, 2016; Ngoh y col., 2017). En comparación con los resultados obtenidos para la quinua, los péptidos formados durante la digestión de las proteínas de amaranto mostraron menor actividad inhibidora de la α-amilasa.

Los digeridos también fueron analizados en relación a sus efectos citotóxicos sobre células de cáncer de colon Caco-2. El digerido gástrico mostró dichos efectos de una manera dosis-dependiente, siendo los péptidos liberados tras la acción de la pepsina los principales responsables de dichos efectos ya que el correspondiente blanco de digestión no presentó actividad a 4 mg/mL, mientras que el digerido inhibió el 80,15% de la viabilidad a esta concentración. El IC_{50} calculado para este digerido fue 0,72 ± 0,01 mg proteína/mL, disminuyendo significativamente tras la acción de la pancreatina hasta valores de IC_{50} de 0,08 ± 0,002 mg proteína/mL y 0,14 ± 0,01 mg proteína/mL a los 60 y 120 minutos de digestión intestinal. Estos resultados indican que los péptidos más potentes que inhiben la viabilidad de las células Caco-2 se liberaron durante los primeros 60 minutos de incubación con la pancreatina, aunque no se puede descartar la contribución de esta enzima y las sales biliares sobre la actividad citotóxica

observada, ya que los correspondientes blancos de digestión mostraron un valor de IC_{50} de 1,24 ± 0,04 mg/mL.

Con el fin de determinar la influencia del peso molecular de los péptidos sobre la multifuncionalidad de los digeridos gastroduodenales, los digeridos gastrointestinales fueron sometidos a ultrafiltración para separar las fracciones peptídicas de peso molecular > 5 kDa y < 5 kDa, midiendo las diferentes actividades biológicas. Los péptidos de bajo peso molecular de los digeridos gastroduodenales fueron los principales responsables de las actividades antioxidante e inhibidora de la ECA, DPP-IV y α-amilasa. En el caso de la actividad inhibidora de la ECA, los valores de IC_{50} de las fracciones conteniendo los péptidos más pequeños fueron significativamente más bajos que los determinados en las fracciones de mayor tamaño y similares a los de los digeridos completos, indicando que los péptidos pequeños fueron los principales responsables de la actividad de los digeridos. Estos resultados confirman estudios previos que han asociado la actividad inhibitoria de la ECA con péptidos de bajo peso molecular (Aluko, 2015). La fracción < 3 kDa del hidrolizado con termolisina de proteínas de la semilla de cereza presentó mayor actividad que la fracción > 3 kDa (García y col., 2015). Asimismo, se ha descrito que los péptidos de menor peso molecular procedentes tanto de proteínas animales como vegetales tienen una mayor actividad antioxidante (Tang y col., 2009; Liu y col., 2016). La fracción < 5 kDa del digerido duodenal de amaranto a los 60 minutos mostró la mayor capacidad de captación de radicales peroxilo, con un valor de ORAC de 3,02 ± 0,13 μmol Trolox equivalente/mg de proteína, lo que correspondió al 70,5% de la actividad mostrada por el digerido completo. Una ligera actividad neutralizante de radicales peroxilo fue detectada en la fracción > 5 kDa, lo que sugiere o bien que péptidos más grandes pudieron contribuir en cierta medida en la actividad antioxidante del digerido o bien que el proceso de ultrafiltración permitió el paso de péptidos pequeños a dicha fracción contribuyendo a su actividad.

Por otro lado, sólo las fracciones < 5 kDa de los digeridos gastroduodenales de las proteínas de amaranto presentaron inhibición de la α-amilasa pancreática, lo que indica que sólo los péptidos de bajo peso molecular procedentes de dichas proteínas

fueron responsables de esta actividad. De forma similar, péptidos de bajo peso molecular derivados de hidrolizados de judía pinta Protamex han sido descritos como los principales inhibidores de la α-amilasa (Ngoh y Gan, 2016). La mayor actividad fue determinada en la fracción obtenida a partir del digerido intestinal de 120 minutos indicando la necesidad de una incubación prolongada en el tiempo para liberar los péptidos de mayor potencia inhibidora de la α-amilasa. También se ha encontrado que los péptidos de bajo peso molecular procedentes del digerido obtenido al final de la digestión simulada fueron los principales responsables de la actividad inhibitoria de la DPP-IV, aunque no puede descartarse la contribución de los péptidos más grandes.

Al contrario de lo observado para el resto de las actividades biológicas evaluadas, los péptidos de mayor tamaño (> 5 kDa) obtenidos a partir de los digeridos gastroduodenales de amaranto mostraron la mayor potencia para inhibir la viabilidad de las células Caco-2. Estos resultados están de acuerdo con el estudio de González-Montoya y col. (2017) que encontraron que las fracciones de alto peso molecular recogidas a partir de los digeridos de soja germinada fueron las más activas inhibiendo la viabilidad celular de diferentes líneas celulares de cáncer de colon humano. Sin embargo, Kannan y col. (2008) encontraron que la fracción < 5 kDa de hidrolizados de salvado de arroz tenía una mayor actividad anti-cancerígena frente a células HepG-2 y Caco-2 que las fracciones de peso molecular entre 5 y 10 y mayor a 10 kDa.

La identificación de los péptidos potencialmente responsables de las actividades encontradas en el digerido gastroduodenal tras 60 minutos de incubación con la pancreatina se realizó en las dos fracciones cromatográficas recogidas (F-1 y F-2). Ambas fracciones presentaron las cinco actividades evaluadas, siendo la F-2 la más activa excepto como inhibidor de la α-amilasa, cuyo valor de IC_{50} fue 2,8 veces mayor que el mostrado por F-1. Los valores de IC_{50} de la F-2 como inhibidor de la ECA, DPP-IV y de la viabilidad de células Caco-2 fueron entre 1,3 y 4,4 veces más bajos que los determinados para F-1. El valor de ORAC de F-2 fue de 4,47 µmol Trolox equivalente/mg proteína, superior al valor obtenido para F-1 (1,56 µmol Trolox equivalente/mg proteína). Mediante el análisis por HPLC-MS/MS se detectaron diversos compuestos peptídicos con una masa molecular entre 500 y 1500 Da. Sin

embargo, al igual que se ha puesto de manifiesto con la quinua, el bajo número de proteínas de *Amaranthus* secuenciadas y recogidas en la base de datos NCBInr limitó el número de péptidos identificados en el estudio. Se identificaron 13 fragmentos que coincidieron con proteínas de amaranto (globulina 11S, acetolato sintasa, albúmina 1 y poliamina oxidasa), de los cuales cinco estuvieron presentes en F-1 y ocho en F-2. De estos péptidos identificados, tres de ellos, NRPET, HVIKPPS y ASANEPEDEN, estaban dentro de la secuencia de péptidos FNRPETT, HVIKPPSRA e ITASANEPEDN que junto con otros péptidos fueron identificados recientemente por Orsini Delgado y col., (2016). Estos autores, en base a los estudios previos de relación estructura/actividad, sintetizaron algunos de los péptidos identificados, midiendo su actividad antioxidante mediante el método ORAC. El péptido HVIKPPSRA mostró un valor de IC_{50} de 0,57 mg/mL. El modelo cuantitativo QSAR ha demostrado la relación entre las propiedades fisicoquímicas de las regiones C-terminal y N-terminal y la potencia antioxidante, siendo la región C-terminal más relevante que la N-terminal. También se ha encontrado que los aminoácidos hidrofóbicos voluminosos situados en la región carboxi-terminal, los aminoácidos polares/cargados en la posición C1, así como la baja carga electrónica en las posiciones N1 y N2 contribuyen a la actividad antioxidante (Li y Li, 2013). Los aminoácidos triptófano, tirosina, metionina, histidina y fenilalanina son capaces de captar radicales peroxilo (Hernández- Ledesma y col., 2005) y el residuo de leucina mejora la captación de radicales libres por parte de los péptidos que lo contienen (Alemán y col., 2011). En nuestro caso, los péptidos que contienen aminoácidos con cadenas laterales voluminosas y principalmente el péptido que contiene tirosina, podrían ser responsables de la capacidad antioxidante observada.

La actividad inhibidora de la ECA parece estar determinada principalmente por el tetrapéptido C-terminal (Zhou y col., 2013). Además, la presencia en estos péptidos de prolina, aminoácidos ramificados, aromáticos o hidrofóbicos podría mejorar significativamente sus propiedades de inhibición (Aluko, 2015). Entre los péptidos liberados de las proteínas de amaranto durante la fase intestinal, ocho contienen más de dos residuos de leucina, prolina, valina o histidina. Adicionalmente, las secuencias

FLISCLL, SVFDEELS y DFIILE contienen fenilalanina que también podría ser determinante en su potencial para inhibir a la ECA.

Puesto que la prolina en la primera, segunda, tercera o cuarta posición N-terminal es determinante en la función estructural de los péptidos con actividad inhibitoria de la DPP-IV (Pieter, 2006) y que recientes estudios *in silico* han demostrado que los potentes péptidos inhibidores de la DPP-IV contienen un aminoácido de cadena ramificada (leucina, isoleucina o valina) o un residuo aromático con un grupo polar en la cadena lateral (principalmente triptófano) en la posición N-terminal (Nongonierma y FitzGerald, 2014; Tulipano y col., 2015), los péptidos NRPET y VEEGNM, liberados de la globulina 11S y la albúmina 1, respectivamente, podrían ser los principales responsables de la actividad inhibidora de la DPP-IV mostrada por las fracciones de amaranto. Sin embargo, otros péptidos contenidos en estas fracciones que no han sido identificados, así como sus potenciales interacciones, también podrían tener una influencia positiva.

Recientemente, se ha descrito que las interacciones aromático-aromático (enlaces de hidrógeno, interacciones electroestáticas y de Van der Waals) entre péptidos derivados de los alimentos y los residuos situados en el centro activo de la enzima son cruciales para la inhibición de la α-amilasa (Siow y col., 2017). Por lo tanto, se ha establecido que la presencia de residuos aromáticos tales como fenilalanina, triptófano y tirosina es una característica estructural de los péptidos inhibidores de esta enzima (Ngoh y col., 2017; Siow y Gan, 2016). Previamente, péptidos derivados de las proteínas de judía pinta y que contenían histidina, metionina, leucina, prolina y glicina demostraron ser buenos inhibidores de la α-amilasa (Ngoh y Gan, 2016; Ngoh y col., 2016). La posición de estos aminoácidos en la secuencia peptídica también juega un papel importante en la inhibición de la α-amilasa (Ngoh y Gan, 2016). La mayoría de los péptidos identificados en F-1 (YESGSQ, GGEDE y NRPET) y F-2 (FLISLL, TALEPT, HVIKPPS, SVFDEELS, ASANEPDEN y DFIILE) presentaban, en los extremos amino y carboxilo terminal, residuos de aminoácidos involucrados en la unión a enzimas.

De manera que considerando en conjunto todos los resultados y basándonos en la relación entre la estructura de los péptidos identificados y las diferentes actividades biológicas, puede concluirse que los péptidos identificados en la fracción F-2, cuyas secuencias fueron FLISCLL, SVFDEELS y DFIILE, tienen un gran potencial para ser considerados como péptidos multifuncionales por su capacidad antioxidante y la capacidad para inhibir la ECA y la α-amilasa. También puede considerarse como péptidos multifuncionales, el péptido NRPET (F-1) como inhibidor de la DPP-IV y la α-amilasa, y el péptido HVIKPPS (F-2) como antioxidante e inhibidor de la α-amilasa. Sin embargo, los hallazgos de este estudio deben ser validados usando péptidos sintéticos individuales que confirmen su multifuncionalidad y permitan evaluar otros parámetros como su biodisponibilidad y mecanismos de acción.

En cuanto a la capacidad inhibitoria de la peroxidación lipídica de los digeridos gástricos y gastroduodenales, ésta se evaluó mediante el método TBARS *in vitro* (Publicación VII). No se observó actividad para el agua destilada utilizada como control negativo, mientras que el BHT (0,2 mg/mL), utilizado como control positivo, inhibió TBARS hasta un 93,4%. No se observó actividad para el concentrado sin digerir. Sin embargo, tras la fase gástrica, los porcentajes de inhibición de TBARS fueron 89,8%, 77,5% y 78,7% para los digeridos obtenidos a pH 1,2, 2,0 y pH 3,2, respectivamente. La mayor hidrólisis de las proteínas de amaranto al pH óptimo de la pepsina pudo ser responsable de la mayor actividad antioxidante determinada en el digerido obtenido a pH 1,2. Además, durante la fase duodenal, la mayor proteólisis producida tras la acción de la pancreatina favoreció la capacidad antioxidante del digerido hasta el 94,9% de inhibición de TBARS, un valor más alto que el observado para el antioxidante sintético BHT. El modelo del pez cebra fue empleado para confirmar la capacidad antioxidante de los digeridos. El valor de inhibición obtenido para BHT fue del 90,0%, mientras que el agua y el CPA sin digerir no mostraron ninguna actividad. Sin embargo, e igual que se había observado con el método *in vitro,* el digerido gástrico obtenido a pH 1,2 presentó la mayor capacidad antioxidante con un porcentaje de inhibición de TBARS del 67,6%. Los digeridos obtenidos a pH 2,0 y pH 3,2 mostraron valores de inhibición de 59,8% y 42,3%, respectivamente. Finalmente, el digerido obtenido después de la

incubación secuencial con pepsina y pancreatina resultó ser el más activo, inhibiendo TBARS en un 77,6%. Con estos resultados, podemos concluir que los péptidos de bajo peso molecular liberados durante las dos fases de la digestión gastrointestinal simulada, podrían ser los responsables de la mayor actividad observada, de manera similar a lo observado para la actividad neutralizante de radicales peroxilo.

4. CONCLUSIONES

CONCLUSIONES

1. Se han optimizado las condiciones del proceso de extracción de los concentrados proteicos de quinua y amaranto, logrando el mayor rendimiento y contenido proteico mediante solubilización de las proteínas a pH 8,0 y posterior precipitación de las mismas a pH 4,0.

2. Durante la fase gástrica del proceso simulado de digestión gastrointestinal, la pepsina degrada de forma parcial las proteínas de quinua y amaranto, que son hidrolizadas completamente tras la acción de la pancreatina durante los primeros 60 minutos de fase duodenal.

3. La digestión gastrointestinal simulada favorece la liberación, a partir de las proteínas de quinua y amaranto, de péptidos antioxidantes, inhibidores de la enzima convertidora de angiotensina, dipeptidil peptidasa IV, α-amilasa y α-glucosidasa y péptidos citotóxicos frente a células de cáncer de colon.

4. Los péptidos de pequeño tamaño liberados durante la digestión gastrointestinal contribuyeron en mayor medida en la actividad antioxidante e inhibidora de las enzimas implicadas en la modulación de la tensión arterial y los procesos metabólicos de los digeridos, mientras que los péptidos de mayor tamaño fueron los principales responsables de la actividad inhibidora de la viabilidad de las células de cáncer de colon.

5. A partir del digerido gastroduodenal obtenido tras 60 minutos de fase intestinal se han identificado 17 nuevas secuencias peptídicas procedentes de las proteínas de quinua. De ellas, los péptidos correspondientes a los fragmentos IQAEGGLT f(26-33), DKDYPK f(163-168) y GEHGSDGNV f(193-201) de la globulina 11S, presentaron moderados efectos anti-diabéticos *in vitro*. Además, debido a su composición aminoacídica, los péptidos LWREGM f(124-129), DKDYPK f(163-168) y DVYSPEAG f(313-320) de la globulina 11S, el péptido IFQEYI f(23-28) de la maturasa K y el péptido RELGEWGI f(469-476) pudieron contribuir a los efectos antioxidantes mostrados por el digerido.

6. De los 13 péptidos identificados tras la digestión gastrointestinal simulada de las proteínas de amaranto y en base a la relación estructura/actividad, los fragmentos FLISCLL f(22-28) y SVFDEELS f(402-409) de la globulina 11S y DFIILE f(56-61) de la poliamina oxidasa pueden considerarse como potenciales péptidos capaces de actuar como antioxidantes, anti-diabéticos y anti-hipertensivos, pudiendo ser responsables de la multifuncionalidad ejercida por el digerido gastroduodenal.

7. Se confirma la utilidad del pez cebra como modelo animal para evaluar la actividad inhibidora de la peroxidación lipídica de péptidos liberados a partir de proteínas alimentarias. En dicho modelo, se han confirmado los potentes efectos antioxidantes de los péptidos liberados a partir de las proteínas de quinua y amaranto durante su digestión gastrointestinal simulada.

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6. ANNEXES / ANEXOS

Publicación I: **Antioxidant activity of tocte protein concentrate from (*Juglans neotropica* Diels) and its capacity to inhibit lipid peroxidation in zebrafihs (*Danio rerio*).**

JOURNAL OF FOOD MEASUREMENT & CHARACTERIZATION (*submitted*)

Publicación II: **Isolation of hen egg white lysozyme by cation exchange chromatography, analysis of its digestibility and evaluation of the inhibition lipid peroxidation in the zebrafish model.**

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH, 9 (2016) 345-349

Compuestos bioactivos derivados de amaranto y quinua.

ACTUALIZACIÓN EN NUTRICIÓN, 16 (2015) 18-22

Nutritional and biological value of quinoa (*Chenopodium quinoa* Willd.).

CURRENT OPINION IN FOOD SCIENCE, 14 (2017) 1-6

Antimicrobial and antioxidant peptides obtained from food proteins.

BIOACTIVE PEPTIDES – TYPES, ROLES AND RESEARCH (2017), Ed. Kathryn Powell, Novinka, New York, pp. 37-57

Antioxidant activity of Tocte Protein Concentrate from (*Juglans neotropica* Diels) and its capacity to inhibit lipid peroxidation in zebrafish (*Danio rerio*)

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ABSTRACT

Tocte Protein Concentrate (TPC) was analyzed with the SDS-PAGE electrophoresis technic. TPC showed eleven bands, out of which three bands showed high expression with molecular weights of 20, 35 and 50 kDa. Three groups of bands were clear with molecular weights ranging from 6.5 to 50 kDa. Protein content of TPC was determined using the Dumas method. At pH 3.0 TPC presents 68.84% content of protein, at pH TPC 4.0 presents 65.77% content of protein, at pH 5.0 TPC presents 73.13% of protein content and at pH 6.0 TPC presents 46.77% of protein content. TPC at pH 3.0, pH 4.0, pH 5.0 and pH 6.0 showed antioxidant activity with the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) method with values of 108.2, 99.0, 33.4 and 74.3 µmol of Trolox/ g tocte proteins, respectively. TPC at pH 3.0 showed the highest value using the oxygen radical absorbance capacity -fluorescein method, 0.73 µmol TE/ mg protein. When the thiobarbituric acid reactive substances (TBARS) method was used in zebrafish larvae, all tocte proteins showed lipid peroxidation inhibition. TPC at pHs 3.0; pH 4.0; pH 5.0 and pH 6.0 at a concentration of 2.5 mg/mL showed a lipid peroxidation inhibition percentage of 40.83%; 50.30%; 54.65%, 78.96%, respectively. Positive control butylated hydroxytoluene, showed 85% of lipid peroxidation inhibition in the zebrafish larvae. None of the proteins from tocte were toxic in zebrafish larvae at the concentration assayed (2.5 mg/mL).

Keywords: tocte, *Juglans neotropica* Diels, antioxidant activity, tocte protein concentrate*, inhibition of lipid peroxidation, zebrafish larvae*

INTRODUCTION

Plant proteins play an important role in human nutrition, especially in developing countries where protein intake is less than the recommended dose [1].

Genus *Juglans* also known as walnut has about 23 species distributed in North, Central and South America, Eastern Europe and Asia. The two most important species are economically the Persian walnut (*Juglans regia*) appreciated for the quality of its nuts and the black walnut (*Juglans nigra*) highly prized for the quality of its timber [2]. In South America, there are the following species: Argentinian walnut (*Juglans australis*), Bolivian walnut (*Juglans boliviana*) and black cedar (*Juglans neotropica*) found in Colombia, Venezuela, Peru and Ecuador and known as tocte. It is known that a living system possesses several natural defense mechanisms, such as antioxidants and enzymes. Oxidative stress resulting of the imbalance of oxidant/antioxidant homeostasis still leads to the generation of reactive oxygen species (ROS), which cause extensive damage to lipids, proteins and DNA [3]. Particularly, the brain is fragile to oxidative stress damage because of its high-energy use, high metabolic demands, high cellular contents of lipids and protein, and low levels of endogenous scavengers [4].

Lipid peroxidation in biological systems has been thought to be a toxicological phenomenon leading to various pathological consequences. Malondialdehyde (MDA) formed from lipid peroxidation of unsaturated phospholipid reacts with thiobarbituric acid (TBA) to produce pink MDA-TBA products. MDA is reactive and active in crosslinking with DNA and proteins and damages liver cells [5]. Phospholipids are believed to be present in high amounts in cell membranes [6]. Lipid peroxidation has been a major contributor to the loss of cell function under oxidative stress [7,8]. The aim of this work was to obtaining protein concentrates of *Juglans neotropica* Diels to

evaluate the *in vitro* antioxidant activity of TPC and its inhibition of lipid peroxidation *in vivo* in the zebrafish larvae model and its digestibility.

MATERIALS AND METHODS

Tocte Protein Concentrate (TPC)

TPC was prepared according to the process described by Vilcacundo et al. (2017) [9]. Defatted tocte flour (DTF) was suspended in water (1:10 w/v) and was adjusted to pH 8.0 with 1 M NaOH. The slurry was centrifuged at 5,000 x *g* for 30 min at 25°C in an Eppendorf centrifuge (USA). The insoluble tocte protein pellet was re-slurried with pH adjusted distilled water as above and centrifuged again. The supernatants mixed together were adjusted to pH 3.0; 4.0; 5.0 and 6.0 kept for 48 h at 4°C with 1 M HCl and subsequently centrifuged at 5,000 x *g* for 30 min. The precipitate was neutralized to pH 7.0 with 1 M NaOH, the samples were dialyzed with a membrane of MWCO of 5 KDa Spectra/ Por (Spectrum Labs, USA) to remove residual salts and phenolic compounds, then lyophilized. Protein content was determined with the Dumas method [10].

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

SDS–PAGE electrophoresis of TPC was carried out according to the method proposed by Laemmli (1970) [11] with 8% and 12% polyacrylamide gel in a Mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, USA). Polypeptide bands were stained in Coomassie Brilliant Blue G-250 during 12 h. Relative molecular masses of protein were determined by a comparison to molecular weight markers (Bio-Rad, Hercules, CA, USA) and using the software Quantity One of Chemidoc (Bio-Rad).

Radical Scavenging Activity ABTS^{+}

ABTS radical scavenging activity was assayed according to the method of Benjakul *et al.* (2012) [12]. The stock solutions included a 7.4 mM ABTS solution and a 2.6 mM potassium persulphate (K_2SO_8) solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL ABTS solution with 50 ml of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a spectrophotometer (Thermo Fisher Scientific Evolution 200 UV/Vis, Waltham, MA USA). A fresh ABTS solution was prepared for all assays. Sample (150 µl) was mixed with 2.850 µl of ABTS reactive and the mixture was left at room temperature for 2 h in the dark. Sample blank was prepared in the same manner except that methanol was used instead of ABTS. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was calculated after sample blank subtraction and was expressed as µmol Trolox equivalents (TE)/ g tocte proteins.

Oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay

The ORAC-FL assay was based on the assay proposed by Dávalos *et al.* (2004) [13]. The reaction was made at 40°C in 75 mM phosphate buffer (pH 7.4). The final solution mixture (200 mL) contained fluorescein (FL) (70 nM), 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) 14 mM, and antioxidant [Trolox (0.2-1.6 nmol) or samples of the tocte proteins (at different concentrations)]. The fluorescence was measured for 137 min (104 cycles). A FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters was used. The instrument was controlled by the FLUOstar Control software version (1.32 R2) for fluorescence measurement. Black polystyrene 96-well microplates (Nunc,

Denmark) were used. AAPH and Trolox solutions were prepared for each assay and FL was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4). All reaction mixtures were prepared in triplicate and at least three independent runs were performed for each sample. ORAC-FL values were expressed as μmol of Trolox equivalent/ mg of protein.

***In vitro* thiobarbituric acid reactive substances (TBARS)**

TPC obtained at pH 3.0; 4.0; 50 and 6.0 were used to evaluate the inhibition of lipid peroxidation [14]. 0.5 g of olive oil was oxidized by heat at 65 °C for 8 days. TPCs were added to obtain concentrations of 2.5 mg/mL, being incubated at 30 °C for 48 h. Butylhydroxytoluene BHT was used as positive control at concentrations of 0.1 mg/mL of BHT. And distilled water was used as negative control. One milliliter of sample was mixed with one milliliter of the 1% thiobarbituric acid (TBA). The solution was heated at 95 °C during 1 h, and cooled down for 15 minutes. Then, absorbance of the final solution containing TPC digests was measured at 532 nm using a spectrophotometer (Thermo Scientific Evolution 200). The decrease of absorbance indicates an increase of antioxidant activity.

The values of antioxidant activity were expressed as the percentage of inhibition of lipid peroxidation as follows: % Inhibition of lipid peroxidation = As/Ab x 100, where Ab is the absorbance of blank and As is the absorbance of the sample.

***In vivo* Thiobarbituric acid reactive substances (TBARS)**

The TBARS method was used as described by Carrillo *et al.* (2016a, 2016b) [15,16]. The group of animals was fed three times a day with food chips for fish. Adult zebrafish were kept for 16 h light and 10 h dark cycles. Embryos were obtained by

photo-induced spawning over green plants and cultured at 28°C in a fish tank water. 5 days post fecundation (dpf) larvae were then incubated in 24-well plates, 30 larvae per well, with 2.5 mg/ml of tocte proteins in each well. Lipid peroxidation was initiated by adding 1 mL 500 µM H_2O_2 and incubated for 8 h at 28°C. Groups of 30 larvae/well in aquarium water were used as controls. BHT was used as positive control and distilled water was used as negative control. Then, H_2O_2 was removed with a micropipette and 500 µl of Tween 0.1% was added. All groups were mixed and homogenized with a T25 Ultra turrax IKA. One milliliter of the solution TBA at 1% was added and subsequently the solution was heated at 95°C for 1 h, then the sample was cooled down during 15 min. Then, absorbance of the solution of zebrafish larvae and peptides was measured at 532 nm. The decrease of absorbance indicates an increase of antioxidant activity (TBARS). The values of antioxidant activity were expressed as the percentage of inhibition of lipid peroxidation as follows: % Inhibition of lipid peroxidation = As/Ab x 100, where Ab is the absorbance of blank and As is the absorbance of the sample.

Test of Toxicity in the Zebrafish Larvae Model

The zebrafish colony was established in the laboratory, in an environmental growth or glass aquarium, provided with an internal filter and aerator activated carbon for water oxygenation. The population of animals was fed three times a day with food chips (Tetra S.A, Melle, Germany) for fish. Adult fish were kept on 16 h light and 8 h dark cycles. Embryos were obtained by photo-induced spawning over green plants and were cultured at 28 °C in fish tank water. Early larvae post-fertilization Zebrafish were maintained according to Kimmel et al. [17]. Larvae of 5 days post fecundation (dpf) were then incubated in 24-well plates, 30 larvae per well for each sample of TPC at 0.4 mg/mL. The volume of fish tank water was 200 µL/well. The effect of each dilution

placed in wells of 200 μL, with 30 larvae/well, was measured at 1, 24 and 36 h from incubation. After 48 h of treatment, the mortality as well as the morphologic changes were assessed. After their respective times, the percentage of larvae death in each dilution was determined. This percentage was plotted versus time. Groups from 30 larvae/well in aquarium water were used as controls. Stereoscopic microscope images were taken to obtain registration expressing the morphological effects on larvae anatomy, as compared to controls. Larvaes were observed at 24 and 48 h under a stereomicroscope (magnification used in the stereomicroscope for observations was 40× using stereomicroscope Motic SMZ8 with camera Moticam 5 MP (Hong Kong, China).

Statistical analysis

Results are presented as means ± standard deviation from three replicates of each experiment. Differences between mean values were determined by the analysis of variance (ANOVA). The post hoc analysis was performed by the Tukey test. All tests were considered significant at $P<0.05$. Statistical analysis was performed using the software package Prism 4 for Windows, version 4.3 (GraphPad Software Inc., www.graphpad.com).

RESULTS AND DISCUSSION

SDS-PAGE electrophoresis analysis

In this study for the first time the proteins from tocte were characterized with the SDS-PAGE method. TPC showed eleven bands, out of which three bands showed high expression with molecular weights of 20, 35 and 50 kDa (Figure 1). Three groups of bands were clear with molecular weights ranging from 6.5 to 50 kDa. Those three groups of bands were clearly stained with coomassie at reducing conditions. These results of *Juglans neotropica* Diels were similar to the ones reported by Mao *et al.* (2012) [18] to *Juglans regia* L with molecular weights of 40

kDa, 35 kDa, 23 kDa and 20 kDa. Both species have then a similar profile of proteins obtained with the alkaline extraction isoelectric precipitation method. TPC at pH 5.0 presented the highest expression of proteins, as those proteins were strongly stained with coomassie.

Protein content of TPC was determined using the Dumas method. At pH 3.0 TPC present 68.84% of protein content, at pH 4.0 TPC present 65.77% of protein content, at pH 5.0 TPC present 73.13% of protein content and at pH 6.0 TPC present 46.77% of protein content. All TPC present high content of protein, being the best percentage the one obtained at pH 5.0 with 73.13% of protein content.

Radical Scavenging Activity ($ABTS^+$)

ABTS radical scavenging activity of different tocte proteins using various pHs of extraction is presented in Table 1. In general, ABTS radical scavenging activity of TPC was high. In TPC, the extraction pH had an influence on the ABTS radical scavenging activity. Results indicated that TPC obtained at pH 3.0; pH 4.0 and pH 6.0 presented values of 108.2, 99.0 and 74.3 µmol Trolox/ g of tocte proteins. These results show then a high activity applying the ABTS radical scavenging method.

TPC obtained at pH 5.0 only presented 33.4 µmol Trolox/ g of tocte proteins, being this pH the lowest value (Table 1). The statistical analysis indicated that there are differences between groups of protein isolate at different pHs ($P<0.05$). ABTS (2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) can be oxidized to generate a radical cation, $ABTS^+$, that is green in colour and can be measured by absorbance at 734 nm. Antioxidants suppress this reaction by electron donation or radical scavenging, thereby inhibiting the formation of the coloured ABTS radical. The concentration of antioxidant in the test sample is inversely proportional to the ABTS radical formation and absorbance at 734 nm [19].

Wang *et al.* (2016) [20] reported that the walnut protein and hydrolysates of walnut protein from *Juglans regia* L presented a high antioxidant activity. Chen *et al.* (2012) [21] reported hydrolysates and peptides isolate from *Junglas regia* with high antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Tironi and Añón, (2010) [22] reported *Amaranthus mantegazzianus* protein isolates with high antioxidant activity using the ABTS method. Amaranth crop 2006 with a value of IC_{50} of 93.5 µg protein/mL of sample and amaranth crop 2007 with a value of IC_{50} of 91.3 µg protein/mL of sample. Orsini-Delgado *et al.* (2011) [23] reported *Amaranthus mantegazzianus* protein isolates at pH 5.0 with high antioxidant activity using the ABTS method with a value IC_{50} of 10.2 mg protein/ mL of sample.

Antioxidant activity of TPC using ORAC method

Antioxidant activity of TPC obtained at pH 3.0, pH 4.0, pH 5.0 and pH 6.0 was evaluated using the ORAC method. Table 1 shows results of antioxidant activity ORAC. The best sample was TPC obtained at pH 3.0 with 0.73 µmol TE/mg of protein. Next best sample was TPC at pH 6.0 with a value of 0.63 µmol TE/mg of protein. Orsini-Delgado *et al.* (2011) [23] reported that amaranth protein isolates at pH 5.0 present 0.12 µg TE/µg of sample (crop 2006) and amaranth protein isolate at pH 5.0 present 0.19 µg TE/µg of sample (crop 2007).

***In vitro* inhibition of lipid peroxidation of TPC**

TPC obtained at pH 3.0, 4.0, 5.0 and 6.0 were used to inhibit the lipid peroxidation of olive oil. TPC at pH 3.0 present a value of 84.28% of inhibition TBARS, TPC at pH 4.0 present a value of 83.45 % of inhibition TBARS, TPC at pH 5.0 was able of inhibit lipid peroxidation with a value 84.33% and TPC at pH 6.0 present the higher percentage with

a value of 86.61% of inhibition TBARS. Carrillo et al., 2017 [14] reported native casein hydrolysate with a value of 55.55% of inhibition TBARS, heated casein hydrolysate with a value of 58.00% of inhibition TBARS. Whey proteins hydrolysates also were able of inhibit of lipid peroxidation but were less active than casein hydrolysates.

In vivo inhibition of lipid peroxidation in the zebrafish larvae model

The values of percentage inhibition of lipid peroxidation indicated that all TPC were efficient to inhibit the lipid peroxidation in zebrafish larvae. As an example, TPC obtained at pH 6.0 presented a result of 78.96% TBARS inhibition at a concentration of 2.5 mg/mL, this activity was high compared to the activity presented in the positive control (BHT) at 0.1 mg/ml with a value of 85% of inhibition lipid peroxidation (Figure 2A). When we compared the results in vitro TBARS with in vivo TBARS, we can observe that in vitro TBARS percentages of all sample assayed were higher than in vivo TBARS. Zebrafish larvae is a complex model because content of in vivo metabolism of larvae. Vilcacundo et al., 2017 [9] reported gastric and duodenal hydrolysates from quinoa (*Chenopodium quinoa* Willd) with capacity to inhibit lipid peroxidation in zebrafish larvae. Gastric digestion at pH 1.2 present a value of 75.12% and duodenal digestion 82.10% of inhibition TBARS in the zebrafish larvae model. Carrillo et al., 2016 [15] reported hydrolysates from hen egg white lysozyme with a reduction of lipid peroxidation in the zebrafish larvae model. Hydrolysate of commercial isolate lysozyme at pH 1.2 present a value of 82.0 and 84.0% of inhibition of lipid peroxidation in zebrafish larvae. Lysozyme without hydrolysis only presented 21% and 23% of inhibition TBARS in zebrafish larvae. Carrillo et al., 2017 reported native and heated hydrolysates from cow milk with capacity to inhibit lipid peroxidation in vivo in the zebrafish model. Previous studies have reported the ability of peptides derived from

different food sources to inhibit lipid peroxidation. As an example, Chen et al. (1998) reported this ability for fish and shellfish protein hydrolysates [24]. Even though the exact mechanism of peptides to act as antioxidant is not clearly known, some aromatic amino acids and histidine are reported to play a vital role in this activity [25,26]. To determine oxidative stress, lipid peroxidation inhibition in the zebrafish larvae model was used to determine cellular damage *in vivo*. Figure 2-B presents the inhibition of lipid peroxidation by TPC at a concentration of 2.5 mg/mL. This assay confirmed that TPC were not toxic for zebrafish larvae. Zebrafish larvae presented a normal aspect after 24 hours of assay.

When zebrafish larvae were examined, no morphological abnormalities were shown such as crooked bodies, spinal deformities or any other significant effects in the growth of the body (Figure 2-B).

CONCLUSION

This study showed the antioxidant activity of TPC (*Juglans neotropica* Diels) cultivated in Ecuador. This work shows for the first time, the capacity of TPC to inhibit lipid peroxidation in the zebrafish larvae model. Proteins from Tocte (*Juglans neotropica* Diels) can be a good source of bioactive components which can be used in functional foods and in medical components. Also, proteins from tocte can be used to reduce the lipid oxidation in the food industry.

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Conflict of Interests: The authors declare that there are no conflicts of interest.

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List of figures and tables

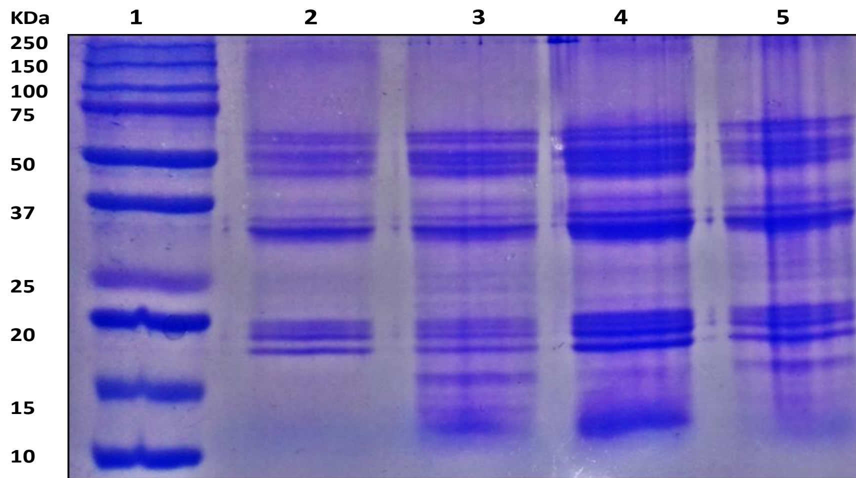

Figure 1. Electrophoresis SDS-PAGE profiles of TPC obtained at different pHs of precipitation. Under reducing condition (2-mercaptoethanol). MW molecular weight marker.

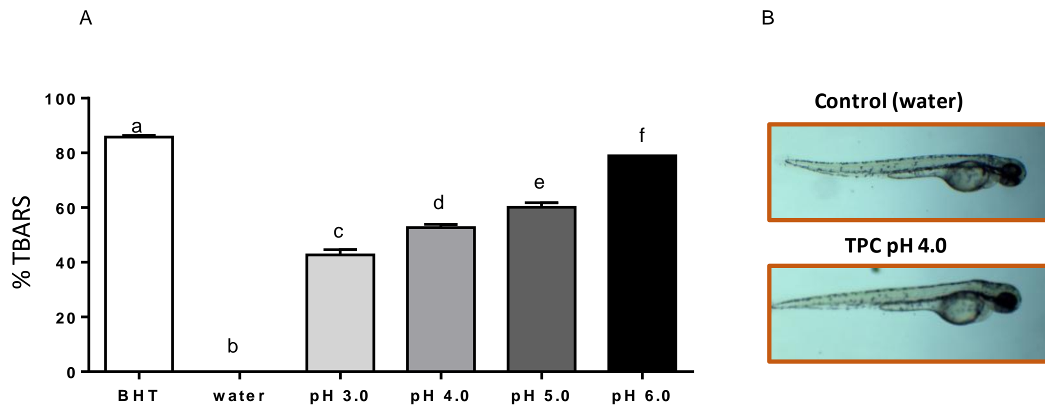

Figure 2. A) TBARS activity in zebrafish larvae model of TPC, B) Test of toxicity of TPC against zebrafish larvae. Results represent the average of three determinations ± SD, values in the same column with different letters are significantly different (P <0.05) (n=3).

Table 1. Antioxidant activity of TPC hydrolysates using ABTS, ORAC and TBARS methods.

Sample	ABTS (µmol TE/g protein) ± SD	ORAC (µmol TE/mg protein) ± SD	% TBARS In vitro
TPC pH 3.0	108.0 ± 0.01[a]	0.73 ± 0.04[a]	84.28± 0.02[a]
TPC pH 4.0	99.01 ± 0.03[a]	0.29 ± 0.00[b]	83.45± 0.02[b]
TPC pH 5.0	33.0 ± 0.02[b]	0.28 ± 0.02[b]	84.33± 0.03[a]
TPC pH 6.0	74.0 ± 0.00[c]	0.63 ± 0.03[c]	86.61± 0.01[c]
BHT			94.12±0.01[d]

Results represent the average of three determinations ± SD, values in the same column with different letters are significantly different (P <0.05) (n=3).

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Research Article

ISOLATION OF HEN EGG WHITE LYSOZYME BY CATION EXCHANGE CHROMATOGRAPHY, ANALYSIS OF ITS DIGESTIBILITY AND EVALUATION OF THE INHIBITION LIPID PEROXIDATION IN THE ZEBRAFISH MODEL

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ABSTRACT

Objective: The aim of this study was to separate and identify lysozyme using cation exchange chromatography, evaluate the protein digestibility and analyze the inhibition lipid peroxidation in the zebrafish model.

Methods: Hen egg white lysozyme was isolate with cation exchange chromatography. Residual muramidase activity was evaluated with the spectrophotometric method. Isolate lysozyme (ILZ) and hydrolysates were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and reversed-phase high-performance liquid chromatography (RP-HPLC).

Results: To identify the protein, the sample was isolated with cationic exchange chromatography, sample was analyzed using RP-HPLC and SDS-PAGE confirming that the fraction was hen lysozyme egg white. The enzymatic activity of the isolated protein was normal compared to the commercial lysozyme activity. Hydrolysates had no muramidase activity and were able to inhibit lipid peroxidation in zebrafish larvae.

Conclusions: Cation exchange chromatography is a good method to ILZ from egg white. Hydrolysates of lysozyme were effective to inhibit lipid peroxidation in zebrafish model.

Keywords: Lysozyme, Cation exchange chromatography, Enzymatic hydrolysis, Muramidase activity.

INTRODUCTION

Lysozyme (EC 3.2.17, N-acetyl-muramic-hydrolase) is a globular basic protein found in nature and is characterized by its high enzymatic activity. It was first discovered in nasal mucous by Alexander Fleming, who named it "Lysozyme" as he observed its lytic activity toward bacterial cocci [1]. The egg albumen is known to have an exceptionally high amount of lysozyme, normally referred to as hen's egg lysozyme, representing 3.5% of the egg white protein content [2-5]. The lysozyme is a basic protein consisting of 129 amino acids and a molecular weight of 14.3 kDa. This enzyme acts by lysing the cell walls of certain Gram-positive bacteria such as lactic acid bacteria and *Clostridium* sp. by splitting β (1-4) linkages between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan of bacterial cell walls [6-9]. Lysozyme has been associated with many biological activities such as antibacterial, antiviral, immunomodulating, immunostimulating, antioxidant, antiviral, and antitumoral activities among others [10-17]. Classical separation methods of proteins are based on the salting out of solution or precipitation with alcohol. These techniques have been extended by the ion exchange (IEX) chromatography and membrane separation in recent years. Presently, researchers are looking for separation methods, which will be cheap, easy, nontoxic, and maintaining the highest biological activity of isolated proteins [18-21]. The aim of the study was to isolate lysozyme (ILZ) using cation exchange chromatography, evaluate its digestibility and then measure their muramidase activity and ability to inhibit lipid peroxidation in zebrafish larvae.

METHODS

Lysozyme and materials

Lysozyme (L2879, chloride form from chicken egg white Grade VI, 40000 units/mg protein, EC 3.2.1.17) and pepsin crystalline (4500 units/mg obtained from porcine stomach mucus and *Micrococcus lysodeikticus*) were purchased from Sigma Chemical Co. (Saint Louis, MO, USA).

Isolation of proteins from egg white

About 500 ml of egg white treated with ethanol 30% and adjusted pH at 5.8 to separate the mucine. The solution was centrifuged at 4.000 rpm for 30 minutes at 4°C. The supernatant was discarded, and the precipitate was adjusted at pH 7.4 with 1 M NaOH and subject to IEX chromatography.

IEX chromatography

Proteins form egg white were separated by IEX as described by Recio and Visser [22] with some modifications using a fast protein liquid chromatography (FPLC) system with a cation exchange column HiLoadTM 26/10 SP-Sepharose Fast Flow (Pharmacia) [23]. Solvent A was 10 mM ammonium hydrogen carbonate acidified to pH 7 with formic acid, and solvent B was 3 M of ammonia solution. The effluent was monitored at 280 nm. Each chromatographic run was repeated 10 times, and the collected fractions were pooled, frozen, and lyophilized. Following these fractions were analyzed with reversed-phase high-performance liquid chromatography (RP-HPLC) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzymatic hydrolysis of lysozyme

Commercial, ILZ and hydrolysates were initially dissolved at 5 mg/ml in potassium phosphate buffer 10 mM (pH 1.5). 1 ml of this lysozyme solution was mixed with 50 mL of pepsin solution of 200 U/mg (5 mg/ml in solution of 0.035 M NaCl, pH 2.0) to obtain an enzyme-to-substrate ratio of 1:20 w/w. This mixture was incubated at 37°C for 1 hr. The reaction was stopped by heating at 80°C for 15 minutes, and the pH was adjusted at 7.0 by addition of 1 M NaOH [7].

SDS-PAGE analysis

The samples were dissolved in 10 mM Tris-HCl buffer, pH 8, 2.5% SDS, and 10 mM ethylenediaminetetraacetic acid (non-reducing conditions) or the same buffer containing 5% b-mercaptoethanol (reducing conditions), and heated at 95°C for 10 minutes [24]. Analysis by SDS-

PAGE used PhastSystem Electrophoresis apparatus, precast 20% homogeneous gels and PhastGel SDS Buffer Strips (GE HealthCare, Barcelona, Spain), following the electrophoretic and silver staining conditions of the manufacturer.

RP-HPLC analysis

Lysozyme hydrolysates, at a concentration of 2.0 mg/ml, were analyzed using a Hi-Pore® RP-318 (250 mm × 4.6 mm i.d.) column (Waters, Milford, MA) in a Waters 600 HPLC system. Solvent A was 0.37% (v/v) trifluoroacetic acid (Scharlau Chemie, Barcelona, Spain) in double-distilled water, and solvent B was 0.27% (v/v) trifluoroacetic acid in HPLC-grade acetonitrile (Lab-Scan, Gliwice, Poland). The chromatographic conditions were as in Martos *et al*. [25]. Detection was at 220 nm, and data were processed using Empower 2 Software (Waters).

Muramidase activity assay

The lytic activity of lysozyme was determined by monitoring the decrease in turbidity of a suspension of *M. lysodeikticus* cell spectrophotometrically at 450 nm at 25°C, according to Shugar's method [26]. One unit of lysozyme was defined as a decrease in the absorbance at 450 nm of 0.001 minutes^{-1}. The muramidase activity of each sample was assayed in triplicate.

Thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid reactive species method was used as described by Westerfield, 1995 [27]. The zebrafish colony was established in the laboratory, in an environmental growth or glass aquarium, provided with internal filter and aerator activated carbon for water oxygenation. The population of animals was fed three times a day with food chips for fish. Adult fish were kept on 16 hrs light and 10 hrs dark cycles. Embryos were obtained by photo-induced spawning over green plants and were cultured at 28°C in fish tank water. Larvae of 5 dpf were then incubated in 24-well plates, 30 larvae per well for each 100 mg/ml of lysozymes and hydrolysates. Lipid peroxidation was initiated by adding 1 ml 500 µM H_2O_2 and incubated for 24 hrs at 28°C. Groups from 30 larvae/well in aquarium water were used as controls. Then, it was removed the H_2O_2, and it was added 500 µl of Tween 0.1%. Each group was mixed and homogenize with a T25 ultra turrax IKA after the absorbance was measured at 532 nm, and the decrease of absorbance indicates an increase of antioxidant activity. Values of antioxidant activity were expressed as the percentage inhibition of lipid peroxidation in larvae homogenate as follows: The total antioxidant activity % inhibition of lipid peroxidation=[(Ab−As)/Ab×100] where Ab = absorbance of control and As= absorbance of sample. The test larval was monitored using a microscope with a Motic Moticam 580, 5 MP.

Test of toxicity in the model of zebrafish

Zebrafish of the AB strain (wild-type, wt) embryos were obtained from natural spawnings. Embryos were raised and fish were maintained as described by Westerfield [27]. After collection and disinfection, eggs were reared in 24-well microplates with 1 ml of water.

Fish embryo toxicity (FET) test

The assay was based on the OECD draft guideline on FET test [28] and is described in detail by Domingues *et al*. [29]. 10 eggs per treatment (3 replicates) were selected and distributed in 24-well microplates. The test started with newly fertilized eggs exposed to the nominal concentrations of 0; 2.5; 5; 25; 50; 100 mg/ml of lysozymes and hydrolysates run for 2 days. Embryos were observed at 24 and 48 hrs under a stereomicroscope (magnification used for observations was ×40). The following parameters were evaluated as an endpoint and considered lethal if one of them is detected egg coagulation, lack of development somites, lack of tail detachment, and lack of heartbeat [30].

Statistical analysis

Results are presented as means±standard deviation from three replicates of each experiment. Differences between mean values were determined by the analysis of variance (ANOVA). The *post hoc* analysis was performed by the Tukey test. All tests were considered significant at p<0.05. Statistical analysis was performed using the software package Prism 4 for Windows, version 4.3 (GraphPad Software Inc., www.graphpad.com).

RESULTS

Hen egg white lysozyme is a protein with high isoelectric point (pI=10.7) in physiological conditions. At pH 7.0, lysozyme has positive charge but at pH >10.7 has negative charge. The egg white has different proteins such as ovalbumin (pI=4.5), ovotransferrin (pI=6.0), and ovomucin (pI=4.1); these proteins have an isoelectric point under 7.0; therefore, they have a net negative charge. These charge differences according to pI allow separating the white egg through cationic exchange chromatography [19]. This fact can be observed in Fig. 1. At the beginning of the chromatogram, a peak with high absorption can be observed at the wavelength used (220 nm), this peak relates to proteins with negative charge nature, as these proteins are unable to join to the negative charge of the column, resulting in being eluted with the solvent. While lysozyme was found retained in the column by charge affinity, the percentage of ammoniac concentration was increased to gradually increase pH, when pH overcome the pI of lysozyme, lysozyme changes the charge to become negatively charged and being eluded from the column, this fact is reflected at the second peak of the chromatogram with an absorption at the wavelength of 220 nm. To determine if lysozyme was effectively separated, the collected FPLC fraction was subject to RP-HPLC with the aim of confirming with its time retained compared to the time retained of a standard of commercial lysozyme (CLZ), to confirm whether or not effectively is lysozyme.

Fig. 2a shows 38 minutes retained time for a standard lysozyme at the concentration of 2 mg/ml; it was then observed the same retained time for the fraction obtained through fast protein liquid chromatography (FPLC) (Fig. 2b). Both CLZ and the fraction of FPLC were subject to hydrolysis with pepsin at pH 1.2; and it was observed the same behavior for both of them. Pepsin hydrolyzes protein completely at this pH. The products of the hydrolysis are identical in the CLZ and the isolated lysozyme (Fig. 2c and d).

The fraction obtained through FPLC was analyzed by SDS-PAGE. In Fig. 3, it is shown that lane 2 reflects the CLZ, lane 3 to the purified FPLC lysozyme, both bands being at the same height of retain in the gel.

Using data of lane 1, which corresponds to the molecular weight marker, we can see that both bands are at 14.400 Da. Confirming the molecular weight of the purified protein in the FPLC obtained fraction. In lanes number 4 and 5, two bands below 14.400 Da can be observed;

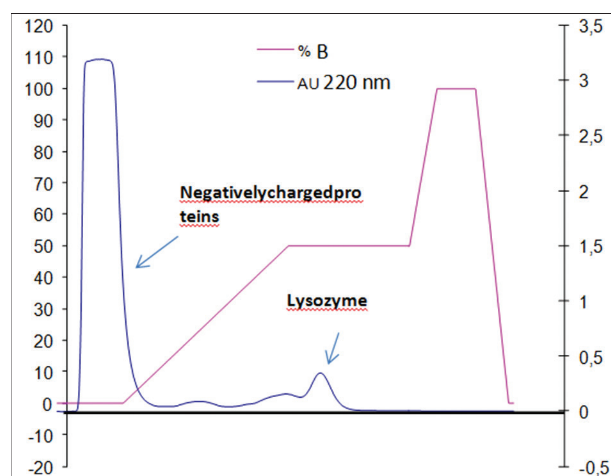

Fig. 1: Separation of lysozyme of egg white by cation exchange chromatography using ammonia 3 M

those lanes relate to peptides resulting of the product of the hydrolysis with pepsin. No intact lysozyme can be observed at this hydrolysis pH.

We finally evaluate the enzymatic activity of the lysozyme of white egg or also called muramidase activity. White egg lysozyme has the capacity of hydrolyzing the walls of bacteria Gram-positive. For this test, lyophilized walls of *Micrococcus lysodeikticus* ATCC 4698 were used, the decrease of observance at 450 nm was measured. In Fig. 4, it can be seen that the FPLC purified lysozyme presented 97% of muramidase activity, the CLZ hydrolyzed only kept 1.7% of said activity and the FPLC purified lysozyme hydrolyzed presented only kept 2.0% of said enzymatic activity, this data indicate that the active site of lysozyme was reduced to small fragments, which have no muramidase activity.

These results are in agreement with different studies where has been described hydrolyzed lysozyme with no muramidase activity [4,31].

In Fig. 5a, it can be seen that CLZ and the isolated lysozyme through chromatography were only able to inhibit the lipid peroxidation in zebrafish larvae 21% and 23%, respectively, whereas hydrolyzed presented a high activity, being able to inhibit 82% for the hydrolyzed of CLZ and 84% for the isolated hydrolyzed lysozyme through cationic exchange chromatography. Both hydrolyzed were effective, and there are not meaningful differences when they are analyzed statistically. The zebrafish larvae after 24 hrs of the assay when were examined no show obvious morphological abnormalities as crooked bodies, spinal deformities, and not exhibit any significant effects on the growth of the body zebrafish larvae (Fig. 5b).

As shown in Fig. 6a, the mortality of the Zebrafish egg when they were treated with different concentrations of lysozymes and hydrolysates. No significant effect on the survival rate was observed for all treatments groups. When the morphologies of these lysozymes treated were examined, no show obvious morphological abnormalities such as egg coagulation, lack of development somites, lack of tail detachment, and lack of heartbeat. The embryos were normal (Fig. 6b).

DISCUSSION

Hen egg is one of the most common foods that induce hypersensitive reactions in young children. Egg white contains more than 20 kinds of proteins. Ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), and lysozyme (Gal d 4) have been identified as main allergens in the egg white. Hen egg white lysozyme is a potent allergen named Gal d4 with resistant at hydrolysis with pepsin. It is known that lysozyme

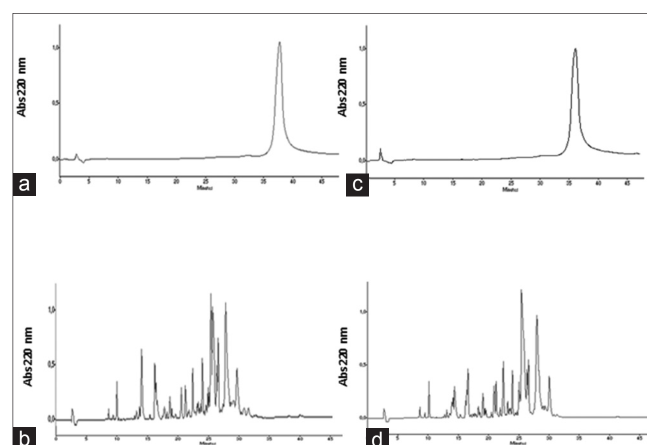

Fig. 2: Reversed-phase high-performance liquid chromatography of lysozyme, (a) commercial lysozyme (CLZ), (b) isolate lysozyme (ILZ), (c) hydrolysate of CLZ, (d) hydrolysate of ILZ

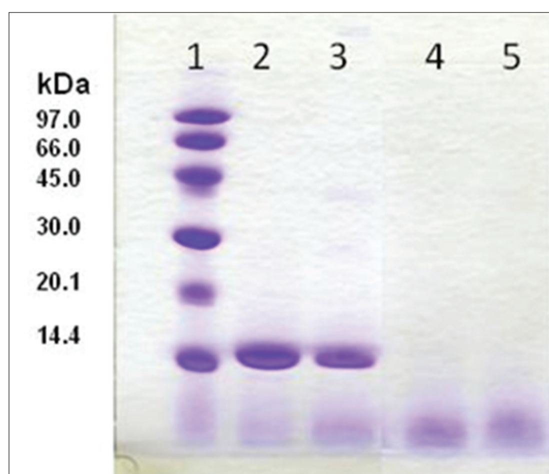

Fig. 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of lysozyme. Lane 1: Weight molecular, Lane 2: Commercial lysozyme (CLZ), Lane 3: Isolate lysozyme (ILZ), Lane 4: Hydrolysate of CLZ, Lane 5: Hydrolysate of ILZ. Coomassie-stained polyacrylamide gel

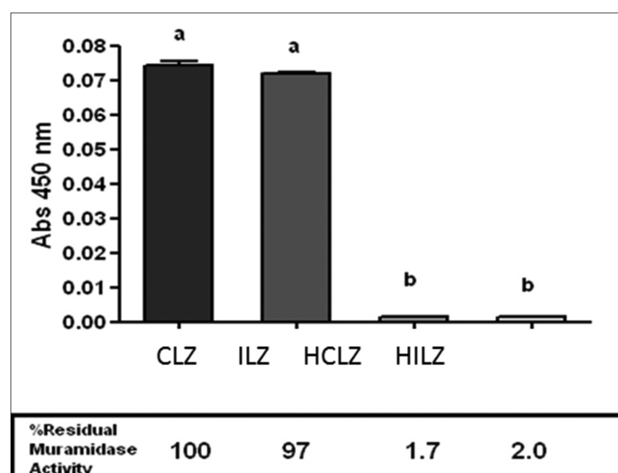

Fig. 4: Residual muramidase activity of commercial lysozyme (CLZ), isolate lysozyme (ILZ), hydrolysate of CLZ and hydrolysate of ILZ. The decrease in turbidity of a cell suspension of *Micrococcus lysodeikticus* was determined at 450 nm in phosphate buffer at pH 6.24 and 25°C. Data were analyzed by analysis of variance (GraphPad Prism), and the means were separated by Tukey's multiple range test. The significance was defined at p≤0.05

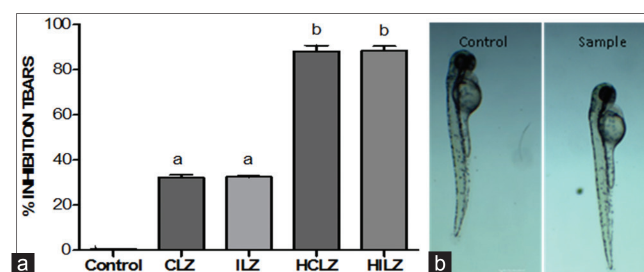

Fig. 5: (a) Thiobarbituric acid reactive results of lysozyme. Data are expressed as % thiobarbituric acid reactive substances inhibition compared to a negative control (error bars expressed as ±SD). n=30 zebrafish larvae. Commercial lysozyme (CLZ), isolate lysozyme (ILZ), hydrolysate of CLZ and hydrolysate of ILZ, (b) Morphologies of zebrafish larvae after incubation with lysozymes

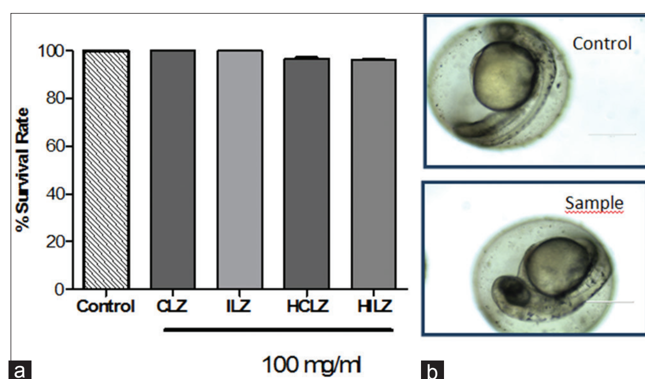

Fig. 6: (a) Survival rate of zebrafish embryos exposed with various concentrations of lysozymes for 48 hrs, (b) photography of zebrafish egg after 48 hrs incubation with lysozymes

has resistance to the hydrolysis with pepsin, but it has been recently described that lysozyme at pH 1.2 has total susceptibility to the hydrolysis with pepsin [7,32-34]. In this study, hen egg white lysozyme was hydrolyzed with pepsin at low pH in simulated gastric fluid (SGF).

The relationship between allergenicity and stability to digestion and the most appropriate experimental conditions for measurement of stability have been the subject of some discussion. The first report by Astwood *et al.* [31] demonstrated that many animals and plant food allergens displayed resistance to pepsin digestion *in vitro*, whereas other common plant proteins believed not to be allergenic were digested rapidly (within 30 seconds). The hydrolysis *in vitro* with pepsin was assayed in SGF 0.35 M NaCl. However, in subsequent studies, the relationship between resistance to digestion and allergenicity was found not to be absolute [35-37].

CONCLUSIONS

Isolate protein by cation exchange chromatography was analyzed with RP-HPLC; SDS-PAGE and identified as hen egg white lysozyme. It slytic enzymatic was normal compared to CLZ. Isolated lysozyme was susceptible to *in vitro* digestion with pepsin at pH 1.5. Hydrolyzed of both commercial and isolated lysozymes were able to inhibit lipid peroxidation in zebrafish larvae.

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ALIMENTOS

COMPUESTOS BIOACTIVOS DERIVADOS DE AMARANTO Y QUINUA

BIOACTIVE COMPONENTS DERIVED FROM AMARANTH AND QUINOA

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RESUMEN
El amaranto y la quinua son pseudocereales reconocidos por la FAO como cultivos importantes en la soberanía alimentaria para la humanidad por sus propiedades nutricionales. Poseen una alta concentración de proteínas las cuales son consideradas de alto valor nutritivo por contener aminoácidos esenciales como la lisina, el triptófano y la metionina. Se han descrito diferentes actividades biológicas de componentes aislados de amaranto y quinua como actividad antibacteriana, antitumoral, antioxidante, antiinflamatoria y antihipertensiva, demostrando que tanto el amaranto como la quinua pueden ser una buena fuente de compuestos bioactivos.
Palabras clave: pseudocereales, compuestos bioactivos, aislados proteicos, proteínas, péptidos.

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ABSTRACT
Amaranth and quinoa are pseudo cereals recognized by the FAO as important crops in food sovereignty for humanity due to their nutritional properties. They have a high concentration of proteins, which are considered highly nutritious as containing essential amino acids such as lysine, tryptophan and methionine. Different biological activities isolated from amaranth and quinoa have been described as antibacterial, antitumor, antioxidant, anti-inflammatory and antihypertensive activity components, demonstrating that both amaranth and quinoa can be a good source of bioactive components.
Key words: *pseudocereal, active components, protein isolate, proteins, peptides.*

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Características funcionales de los pseudocereales amaranto y quinua

El *Amarantus caudatus* o kiwicha en la lengua aborigen quechua y la quinua (*Chenopodium quinoa willd*) son dos pseudocereales de reconocido valor nutricional. Son pseudocereales porque no pertenecen a la familia de las gramíneas; además son dicotiledóneas y los cereales comunes son monocotiledóneas. Estos cultivos ancestrales han sido usados por el hombre desde hace más de 6.000 años[1]. Fueron cultivos muy importantes para culturas precolombinas como los Mayas, Aztecas e Incas. Pero su uso fue decreciendo después de la conquista de los españoles. El amaranto y la quinua son considerados cultivos con una amplia variabilidad genética y una alta capacidad adaptativa a diferentes hábitats agro-climáticos y diferentes tipos de suelos[2,3]. El amaranto posee un alto porcentaje de proteínas, cerca de un 14% con una elevada calidad por su elevado contenido de aminoácidos esenciales como la lisina, el triptófano, la cisteína y la metionina.

Elemento	Quinua rosada	Amaranto	Trigo	Arroz
Proteína	12,5	18	11	6,8
Lisina	6,91	8,0	2,6	3,8
Fenilalanina	3,85	7,7	8,2	10,5
Triptófano	1,28	1,5	1,2	1,1
Metionina	1,98	4,2	3,7	3,6
Isoleucina	6,95	3,7	4,2	4,1
Leucina	6,50	5,7	6,8	8,2
Valina	3,05	4,3	4,4	6,1
Treonina	4,50	3,6	2,8	3,8
Histidina	2,85	2,5	1,7	2,16
Arginina	7,11	10,0	3,6	5,36

Tabla 1: Contenido de aminoácidos en g/100 g de proteína seca. Tabla modificada de FAO, 2013; Pérez et al., 2002[46,49].

En la Tabla 1 se puede observar el perfil de aminoácidos en amaranto y quinua. Sus proteínas tienen mejor calidad que las proteínas de cereales tan comunes como el trigo y el arroz[4,5]. Su calidad se asemeja incluso a proteínas de origen animal como las de leche de vaca y huevo de gallina; además sus proteínas poseen un alto grado de digestibilidad[6]. Las proteínas de amaranto están constituidas principalmente por albúminas que conforman alrededor del 49 al 65% del total, seguidas en abundancia por las glutelinas, 22,4 al 42,3%, las globulinas, 13,7 al 18,1% y finalmente las prolaminas que conforman del 1 al 3,2% del total de las proteínas[7]. Los aislados proteicos de amaranto obtenidos por precipitación poseen una cantidad alta de globulinas en comparación con los otros tipos de proteínas. La fracción de las globulinas a su vez se divide en dos fracciones: la 7; 8S y la 11; 12S llamadas civilina y legumina respectivamente. La globulina 11S se caracteriza por formar hexámeros y ser soluble en soluciones salinas y pH neutro; posee masas moleculares comprendidas en 300 y 360 kDa[8,9]. Los hexámeros están unidos por polipéptidos de diferentes pesos moleculares[10,11]. En los aislados de amaranto se encuentra presente la fracción globulina-P. Esta globulina tiene la particularidad de formar agregados de alto peso molecular (600-1500 kDa)[12] estabilizados por puentes disulfuro. Una proporción baja de las moléculas de globulina-P se encuentra libre, las cuales poseen una masa molecular de alrededor de 300 kDa. La globulina-P posee una alta estabilidad térmica. En los aislados de amaranto hay una cantidad importante de albúminas que se caracterizan por la alta solubilidad. Varios autores coinciden que esta fracción se compone por polipéptidos de masas moleculares de 10 a 40 kDa[5,13]. En la semilla de amaranto se encuentran presentes, además de albúminas y globulinas, las prolaminas y glutelinas características de cereales tales como trigo, cebada y maíz. Las prolaminas son solubles en alcohol y las glutelinas en soluciones de pHs extremos, por lo tanto se las encuentra en baja cantidad en los aislados proteicos de amaranto obtenidos por precipitación isoeléctrica. Las prolaminas están compuestas por polipéptidos de baja masa molecular comprendidos entre 10-22 kDa[14]. También posee minerales, vitaminas y un alto porcentaje de fibra. El género *Amaranthus* pertenece a la familia de las *Amaranthaceae* que incluye cerca de 60 especies distribuidas por todo el mundo[15,16], de las cuales tres se usan para fines alimentarios como son *Amaranthus hypocondriacus*, *Amarantus Caudatus y Amaranthus cruentus*[17].

Los mayores productores de amaranto en la región andina son Bolivia y Perú. Luego están países como Estados Unidos, México, India, Japón y China; también se cultiva considerablemente en diferentes países de África.

La quinua también posee un alto porcentaje de proteínas, cerca de un 15% con una elevada calidad debido a la presencia de los mismos aminoácidos esenciales que se encuentran en el amaranto como lisina, triptófano, cisteína y metionina. Sus proteínas también tienen mejor calidad al ser comparadas con granos de cereales como el trigo y el arroz, además posee muchos minerales, vitaminas y fibra (Tabla 2). Ambos cereales andinos han sido considerados por la FAO como elementos claves para la seguridad alimentaria de la humanidad debido a sus propiedades nutritivas y a su facilidad de adaptación a diferentes climas y altitudes. Son cultivos resistentes que necesitan poco riego y productos químicos para su mantenimiento. Las plagas se controlan con una simple rotación de cultivos. En la década de 1990 la NASA escogió a la quinua como alimento importante para la seguridad alimentaria por su alto valor nutricional. Fue seleccionada para viajes espaciales de larga duración por sus excelentes propiedades nutricionales como su alto contenido de proteínas (12-18%), ricas en aminoácidos esenciales en particular lisina y aminoácidos azufrados. Fue incluida en el programa CELSS por su altura de crecimiento, su fácil manejo y por la capacidad de transformar grandes cantidades de CO2 por tener una fotosíntesis especializada de tipo C4[18].

Elemento	Quinua	Amaranto	Trigo	Arroz	Maíz	Cebada
Proteína %	16,3	12-19	14,2	7,6	10,2	10,8
Grasa %	4,7	6,1-8,1	2,3	2,2	4,7	1,9
Carbohidratos %	76,2	71,8	78,4	80,4	81,1	80,7
Fibra cruda %	4,5	3,5-5,0	2,8	6,4	2,3	4,4
Cenizas %	2,8	3,0-3,3	2,2	3,4	1,7	2,2
Energía (Kcal/100g)	399	391	392	372	408	383

Tabla 2: Características del amaranto y quinua frente a otros cereales. Tabla modificada de Romo et al., 2006[50].

El amaranto y la quinua poseen una serie de antinutrientes como las saponinas, ácido oxálico, taninos, inhibidores de tripsina y ácido fítico. Las saponinas y el ácido fítico son compuestos de naturaleza muy tóxica incluso a concentraciones muy bajas[19]. El ácido fítico se encuentra en gran cantidad en el amaranto y tiene la propiedad de formar complejos

con cationes metálicos como el Mg^{+2}, Fe^{+2}, Fe^{+3} Ca^{+2}, Zn^{+2}, Co^{+2} y el Cu^{+2} ocasionando una pérdida de biodisponibilidad intestinal de estos componentes que traza con la consecuente posibilidad de sufrir desnutrición especialmente en países subdesarrollados donde no están cubiertas las aportaciones necesarias de estos metales importantes[20]. Se han descrito efectos favorables del ácido fitico como actividad antioxidante, prevención de enfermedades cardíacas y actividad anticancerígena[21,22]. Los tratamientos tecnológicos a los que son sometidos los granos pueden reducir el porcentaje de estos antinutrientes. Por el ejemplo un lavado previo con agua de los granos de quinua permite retirar casi en su totalidad las saponinas. Las saponinas son un problema porque le dan un sabor amargo al producto[21]. La quinua se la puede clasificar de acuerdo al contenido de saponinas en: quinua libre (lavada), quinua dulce <0,06% de saponinas y quinua amarga >0,16% de saponinas[22]. El lavado y el tratamiento térmico del tostado de los granos de amaranto permiten reducir el ácido fítico según la especie[23,24].

Actualmente existe una búsqueda de compuestos activos que puedan ejercer su función sobre el organismo; de dichos compuestos la mayoría es de origen alimentario y muchas veces son proteínas o péptidos. Entre los productos más estudiados ricos en proteína que poseen propiedades funcionales se encuentran la soja, la leche y el huevo[25,26]. En estos alimentos se han identificado diferentes péptidos con actividad biológica que pueden ser beneficiosos para la salud y la prevención de enfermedades. Estos péptidos presentan actividad inhibidora de la enzima convertidora de angiotensina (ACE), antimicrobiana, agonistas y antagonistas opioides[26] y antitumoral[27]. Actualmente se están realizando estudios clínicos de péptidos con actividad antitumoral como el inhibidor de tripsina de Bowman & Birk (BBI) y la lunasina[28,29,30]. Es de destacar que estudios epidemiológicos sugieren que una dieta rica en productos a base de soja estaría asociada a una baja incidencia de cáncer, principalmente de mama, colon y próstata[31,32].

En relación al *Amaranthus*, se han descrito péptidos con actividad biológica entre los que se encuentran inhibidores de proteasas y alfa-amilasas[33,34], péptidos antimicrobianos y antifúngicos que poseen un dominio rico en cisteína/glicina característico de proteínas ligadoras de quitina[35,36]. Utilizando herramientas informáticas, se ha sugerido la presencia de péptidos bio-activos en las secuencias de las proteínas de reserva de *Amaranthus hipocondríacus*[37]. Estos péptidos presentarían actividad antitrombótica, inmunomodulatoria, opioide, antioxidante, antihipertensiva e inhibidores de proteasas. Se ha descrito la acción citomoduladora de aislados proteicos de *Amaranthus mantegazzianus* (MPI) en un modelo de células en cultivo; dicho aislado proteico fue activo frente a las cuatro líneas tumorales ensayadas, la línea UMR106 fue la más sensible con un IC_{50} de 1mg/ml. Esta actividad antiproliferativa mejora cuando el aislado proteico fue tratado con proteasas presentando un IC_{50} de 0,5 mg/ml. Los péptidos inhiben la proliferación e inducen la muerte de células de origen tumoral con mayor potencia que las células no tumorales, sugiriendo un potencial efecto antitumoral[38]. Estos resultados in vitro son alentadores para iniciar estudios in vivo y determinar el potencial efecto antitumoral. Se ha descrito actividad reductora de colesterol por parte de las proteínas de *Amaranthus* (este efecto se observó en un modelo animal[39]). Extractos obtenidos de *Amaranthus hypocondriacus* presentaron una fuerte actividad antioxidante con el método de inhibición de DPPH[40]. El grupo de Repo-Carrasco et al.[41] ha descrito actividad antioxidante de dos tipos de *Amaranthus* y determinó la concentración de compuestos fenólicos. Pasko et al.[42] analizaron el contenido de compuestos fenólicos en dos tipos de *Amaranthus* y quinua encontrándose que la quinua presentó mayor actividad antioxidante que el *Amaranthus* debido a la mayor presencia de compuestos fenólicos en la quinua. El grupo de Repo-Carrasco-Valencia et al.[43] también describió una alta concentración de compuestos fenólicos comparado con el *Amaranthus* por ello la actividad antioxidante fue mayor en la quinua. Se ha descrito que extractos fenólicos obtenidos de *Chenopodium quinoa* presentaron una fuerte actividad antioxidante y anticancerígena[44].

Por otro lado, también se ha descrito otro tipo de actividades como efecto antidiabético y anticolesterolémico de extractos en metanol de *Amaranthus caudatus*, *Amaranthus spinosus* y *Amaranthus* viridis en ratas tratadas con streptozotocin para inducirles diabetes, encontrándose que los extractos a la dosis de 400 mg/kg fueron significativamente activos tanto a nivel antidiabético como anticolesterolémico. Mientras que a la dosis de 200 mg/kg sólo presentó actividad antidiabética[45]. Se ha descrito actividad antioxidante y antipirética de extractos metanólicos de *Amaranthus spinosus*. La actividad antioxidante fue evaluada mediante la inhibición de los radicales DPPH, encontrándose una fuerte

actividad antioxidante con valores de IC_{50} de (87,50 ±3,52) µg/mL, (98,80±1,40) µg/mL, (106,2±0,20) µg/mL, (88,7±0,62) µg/mL y (147,50±2,61) µg/mL por DPPH. La actividad antipirética fue estadísticamente significativa (P <0,01)[46]. Este mismo grupo también describió actividad antidiabética, antipirética y antilipídica de extractos metanólicos de *Amaranthus viridis*. Las ratas mostraron una bajada en sangre de glucosa y lípidos. La actividad antioxidante también fue significativa[47]. En conclusión el amaranto y la quinua pueden ser usados como fuente de nuevos compuestos bioactivos: extractos, proteínas, hidrolizados y péptidos que podrían usarse en la industria alimentaria.

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Current Opinion in Food Science

Nutritional and biological value of quinoa (*Chenopodium quinoa* Willd.)

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Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal traditionally consumed by Andean cultures that is attracting attention worldwide as a functional food. Because of its tolerance to extreme environmental conditions and its nutritional and biological properties, quinoa has been defined as 'one of the grains of the 21st century'. In addition to its high content in protein, lipids, fiber, vitamins, and minerals, and its excellent balance of essential amino acids, quinoa has been found to contain numerous phytochemicals including saponins, phytosterols, phytoecdysteroids, phenolics and bioactive peptides. These compounds may exert beneficial effects on metabolic, cardiovascular, and gastrointestinal health. This review summarizes the nutritional and functional role of quinoa emphasizing on the evidence demonstrated by animal and clinical studies.

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Introduction

Quinoa (*Chenopodium quinoa* Willd., Amaranthaceae) is a grain-like food crop traditionally used to provide nutrition and sustenance to Andean indigenous cultures for centuries. Quinoa is mainly grown in Peru, Bolivia, Ecuador, Argentina, Chile, and Colombia, although in the last years, it has been introduced in Europe, North America, and Africa with high yields [1]. The quinoa consumption in high-income countries is increasing although is still low compared with the main producer countries of this plant. Thus, quinoa annual consumption in Bolivia and Peru was of 2.37 kg/person and 1.15 kg/person, respectively, whereas the consumption was of 0.03 kg/person in the US [2]. This plant does not belong to the Gramineae family but it produces seeds that can be milled into flour and used as a cereal crop, thus it is habitually referred to as a pseudo-cereal. A number of toasted and baked goods are produced from quinoa flour, such as bread, cookies, biscuits, noodles, pasta, and pancakes, among others [3]. Moreover, quinoa seeds can be fermented to make beer, or a traditional ceremonial alcoholic beverage from South America called 'chicha' [4]. Quinoa leaves are eaten similarly to spinach [5], and the germinated quinoa seedlings (quinoa sprouts) are incorporated in salads [6]. The whole plant has been also used as a rich nutritional source to feed livestock, including cattle, pigs, and poultry [3].

Because of its stress-tolerant characteristics and its nutritional and biological properties, quinoa has been described, together Amaranth, as 'one of the grains of the 21st century' [7]. Quinoa plant is cold, salt, and drought tolerant, and it can be cultivated in high altitudes in the mountain areas. Moreover, recent investigations have focused on the chemical constituents and therapeutic properties of quinoa that is rapidly gaining recognition as a functional food and nutraceutical [8]. The Food and Agriculture Organization of the United Nations (FAO) launched the International Year of Quinoa in 2013 to promote the production, preservation, and consumption of this crop [9]. This review will summarize the nutritional and biological properties of quinoa emphasizing on the animal and clinical studies performed to demonstrate the health benefits of this crop.

Nutritional properties of quinoa

Table 1 shows a comparison of the nutritional values of quinoa in relation to rice and wheat, considered as some of the most crucial foods worldwide in both human and animal diets. Quinoa's superiority over these and other grains (rye, barley, and oat, among others) results from its richer protein, lipid, and ash content. Protein content (expressed as g/100 g edible matter) of quinoa seeds is ranged between 13.1% and 16.7%. These values are higher than those of rice, barley, corn, and rye, and close to that of wheat [10]. Albumins and globulins represent the major storage quinoa proteins, with percentages of 35% and 37%, respectively. However, prolamins are present in low concentrations [1]. In addition to their high quantity, quinoa proteins are accepted as high-quality protein because of their balanced pattern of essential amino acids (Table 1). All essential amino acids are present in quinoa protein, meeting amino acid

Table 1

Comparison of the nutritional values of quinoa and grains (wheat and rice). Data from Abugoch James, 2009 [1]; Vega-Gálvez et al., 2010 [7]; Hübner & Arendt, 2013 [16]; Navruz-Varli & Sanlier, 2016 [12], and Nowak et al., 2016 [17•].

Nutrient	Quinoa, raw	Wheat	Rice
Energy (kcal)	357–368	340	354
Total protein[a]	13.1–16.7	11.3	6.8
Total fat[a]	5.5–7.4	1.7	0.7
Carbohydrates[a]	59.9–74.7	63.7	79.7
Fiber[a]	7.0–11.7	12.2	0.6
Ash[a]	2.7–3.8	1.5	0.5
Minerals[b]			
Ca	27.5–148.7	35.0	22.0
Fe	1.4–16.7	5.0	1.4
Mg	26.0–502.0	103.0	NA
P	140.0–530.0	393.0	119.0
K	696.7–1475.0	478.0	80.0
Na	11.0–31.0	2.0	31.0
Zn	2.8–4.8	3.7	0.6
Cu	1.0–9.5	0.4	0.1
Vitamins[b]			
Ascorbic acid (C)	4.0–16.4	ND	ND
α-Tocopherol (E)	2.6–5.4	1.4	0.7
Thiamin (B_1)	0.3–0.4	0.5	0.2
Riboflavin (B_2)	0.3–0.4	0.1	0.1
Niacin (B_3)	1.1–1.5	5.1	4.4
Pyridoxine (B_6)	0.5	0.3	0.3
Folate	0.2	0.1	0.1
Essential amino acids[c]			
His	1.4–5.4	2.4	2.4
Ile	0.8–7.4	4.3	4.3
Leu	2.3–9.4	8.3	8.3
Lys	2.4–7.8	3.6	3.6
Met	0.3–9.1	2.4	2.4
Cys	0.1–2.7	2.1	2.0
Phe + Tyr	2.7–10.3	8.7	8.7
Thr	2.1–8.9	3.6	3.6
Trp	0.6–1.9	1.2	1.2
Val	0.8–6.1	6.1	6.1

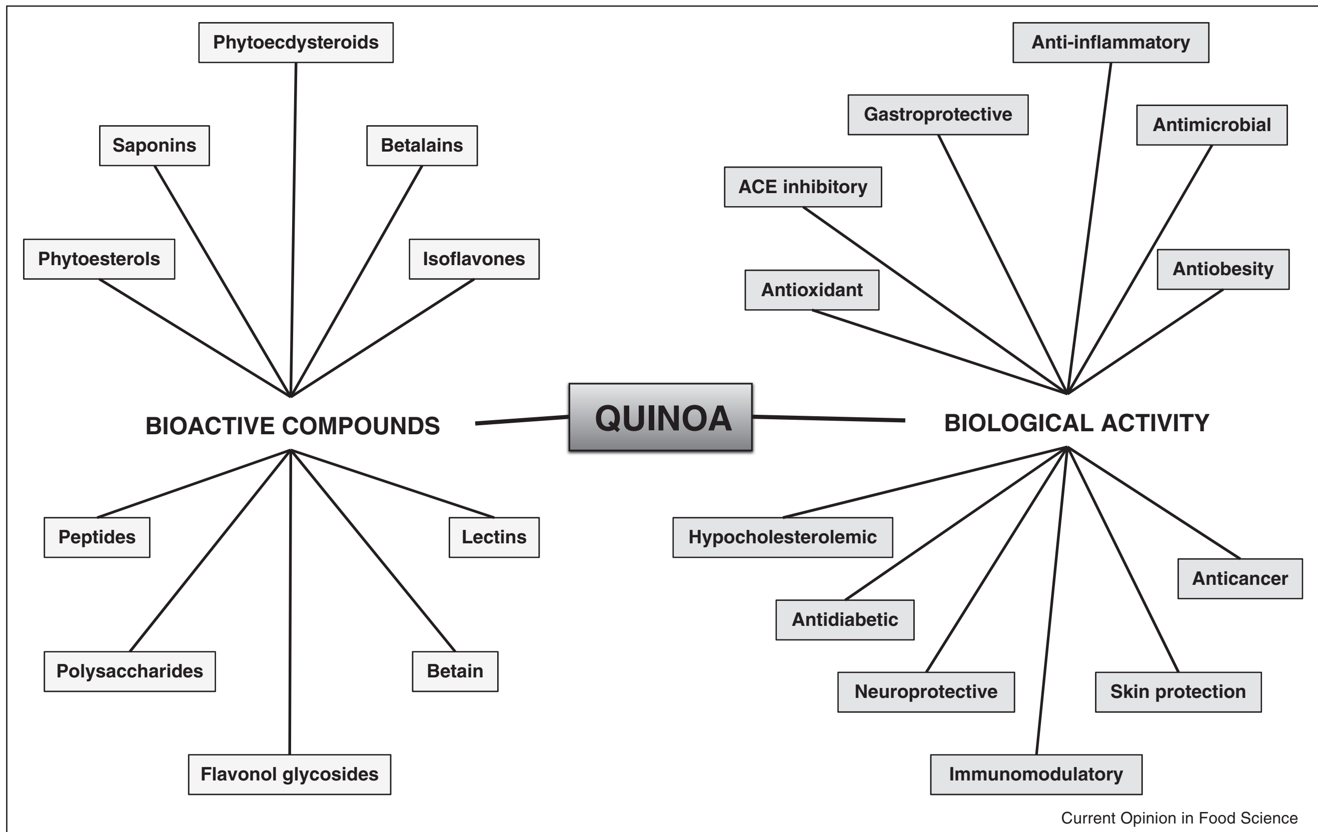
[a] g/100 g edible material.
[b] mg/100 g dry matter.
[c] g/100 g protein.
NA: not available; ND: not detected.

requirements suggested by FAO/WHO/UNU for adults [11]. Quinoa protein presents high content in lysine (ranged from 2.4 to 7.8 g/100 g protein), methionine (0.3–9.1 g/100 g protein), and threonine (2.1–8.9 g/100 g protein) that are the limiting amino acids in conventional cereals, such as wheat and maize [12]. The carbohydrate content of quinoa seed is similar to that of wheat and rice. Starch is the major carbohydrate component constituting 32%-69% of it [1]. Total dietary fiber in quinoa (7.0–11.7 g/100 g edible matter) is near that of cereals such as wheat, while soluble fiber content is ranged from 1.3–6.1 g/100 g edible matter. Individual sugars represent 3% of quinoa seeds, and are mostly maltose, D-galactose and D-ribose in addition to low levels of fructose and glucose [1]. Besides its high content and good biological quality of their proteins, quinoa seed has an interesting lipid composition of about 5.5–7.4 g/100 g edible matter (Table 1), higher than wheat (1.7 g/100 g edible matter) and rice (0.7 g/100 g edible matter), making quinoa be accepted as an alternative oilseed seed [13••]. Palmitic acid is the major saturated fatty acid found in quinoa, constituting 10% of total fatty acids, while unsaturated fatty acids oleic (19.7–29.5%), linoleic (49.0–56.4%), and alpha-linolenic (8.7–11.7%) acids represent 88% of the total fatty acid amount of quinoa seeds, in a similar way to soybean lipid composition [14]. Fatty acids of cell membranes are well protected against damage caused by free radicals by the presence of vitamin E at higher concentration than that of wheat [1,15]. The levels of other vitamins such as riboflavin (B_2), pyridoxine (B_6), and folic acid are also higher than those of most other grains like wheat, rice, barley, and corn. Pyridoxine and folic acid levels in 100 g of quinoa are reported to meet adults' daily requirements while riboflavin meets 80% of children's and 40% of adults' needs [1]. High vitamin C levels have been also determined in quinoa seeds ranged from 4.0 to 16.4 mg/100 g dry matter (Table 1). However, the thiamin content is lower than that of oat and barley [13••]. The mineral content of quinoa is also of great importance. The seeds have high content of calcium, magnesium, iron, copper, and zinc. Many of these minerals are present in higher concentrations to those found in common grains. Moreover, calcium, magnesium, and potassium are found in quinoa in bioavailable forms, thus their contents are considered to be adequate for a balanced diet [8,14].

Functional potential of quinoa for human health

In addition to its high nutritional value and gluten-free attribute, quinoa has been reported to exert beneficial effects on high-risk group consumers, such as children, the elderly, lactose intolerant, and people with anemia, diabetes, obesity, dyslipidemia, and celiac disease. These benefits have been linked with the content of protein, fiber, vitamins and minerals, fatty acids, and especially with the presence of a plethora of phytochemicals that provide quinoa a remarkable advantage over other grains in terms of human nutrition and health [13••]. The bioactive compounds identified in quinoa and their reported biological activities are shown in Figure 1. Quinoa's outer seed coat is rich in bitter saponins that interfere with its palatability and digestibility making needed their removal before seed consumption. Despite their unpalatable characteristics, a wide range of biological activities have been described for saponins, including antifungal, antiviral, anticancer, hypocholesterolemic, hypoglycemic, antithrombotic, diuretic, and anti-inflammatory activities [18••]. The total quinoa saponin fraction has been reported to slightly inhibit the growth of *Candida albicans* [19]. The activity of this fraction against the mycelia growth of *Botrytis cinerea* was improved by alkali treatment, probably due to the formation of more

Figure 1



Bioactive compounds and biological activities described for quinoa (*Chenopodium quinoa* Willd.).

hydrophobic saponin derivatives with higher affinity with the sterols present in cell membranes [20]. Madl and co-workers identified, by nano-HPLC electrospray ionization multi-stage mass spectrometry, 87 triterpene saponins and five novel triterpene aglycones [21]. More recently, Kuljanabhagavad and co-workers identified 20 triterpene saponins from different parts of quinoa plant evaluating their cytotoxic activity in cervix adenocarcinoma HeLa cells [22]. Moreover, a saponin-rich quinoa seed extract has been found to inhibit the release of pro-inflammatory cytokines, and to decrease the production of nitric oxide in lipopolysaccharide-stimulated RAW 264.7 macrophages [23]. The ability of quinoa saponins to affect differentiation of 3T3-L1 preadipocytes and therefore, suppress adipogenesis has also been investigated [24]. Phytosterols are lipophilic compounds structurally similar to cholesterol. Due to this similarity, they compete for cholesterol's intestinal absorption and reduce atherogenic lipoprotein production in the intestines and liver, thus exerting reduction of serum cholesterol levels [25]. In addition, antioxidant, anti-inflammatory, and anticancer activities have been described for phytoesterols [26]. These authors found that quinoa contains higher content of phytoesterols than those in cereals such as barley, rye and corn, with β-sitosterol (63.7 mg/100 g), campesterol (15.6 mg/100 g), and stigmasterol (3.2 mg/100 g) as the predominant components.

One of the main activities demonstrated for quinoa seeds is the antioxidant activity that has been associated with their high content of phenolic compounds [27]. More than 20 phenolic compounds have been found in either free or conjugated forms (liberated by alkaline, acid, and/or enzymatic hydrolysis). Mostly, they are phenolic acids consisting of vanillic and ferulic acids, and their derivatives as well as the flavonoids quercetin, kaempferol, and their glycosides [28,29]. In addition to their antioxidant properties, these quinoa components have been reported to exert α-glucosidase and pancreatic lipase inhibitory activity [29]. Phytoecdysteroids are polyhydroxylated steroids implicated in plant defense because of their structural relationship with insect molting hormones. Moreover, a wide range of health benefits have been demonstrated for these components, including anabolic, performance enhancing, anti-osteoporotic, anti-diabetic, anti-obesity, and wound healing properties [30]. Quinoa is one of the richest edible sources of phytoecdysteroids,

Table 2

Clinical trials on the effect of quinoa products in human health.

Study participants	Treatment	Duration	Outcomes	Conclusions	Reference
50–65-month old boys	100 g quinoa-added baby food	15 days	Increase of IGF-1	Potential role in reducing childhood malnutrition	[36]
22 students (18–45 years old)	Quinoa cereal bars	30 days	Reduction of triglycerides, total cholesterol and LDL levels Reduction (no significant) of blood glucose and pressure, and body weight	Potential role in preventing cardiovascular disease	[37]
35 post-menopausal overweight women	25 g quinoa flakes and cornflakes	4 weeks	Reduction of triglycerides, TBARS and vitamin E Increase of urinary secretion of enterolignans Decrease of total cholesterol and LDL Increase of GSH	Beneficial effect on metabolic parameters modulation	[38]
19 celiac patients	50 g quinoa	6 weeks	Improved histological and serological parameters Mild hypocholesterolemic effect	Quinoa consumption is safe for celiac individuals	[39•]

with a content ranged from 138 to 570 μg/g and 13 different phytoecdysteroid types. Among them, the most common is 20-hydroxyecdysone (20HE) that constitutes 62–90% of total quinoa phytoecdysteroids [18••]. A 20HE-enriched extract obtained from quinoa was demonstrated to reduce fasting blood glucose in obese, hyperglycemic mice [30].

Takao and co-workers reported cholesterol-lowering effects of a quinoa protein-enriched fraction in mice [31]. Moreover, enzymatic hydrolysis has been described as a suitable strategy to release bioactive peptides from quinoa proteins. Aluko and Monu studied the functional (foaming) and biological (antioxidant and angiotensin-converting enzyme inhibitory) properties of an alcalase hydrolyzate of quinoa proteins [32]. Recently, papain hydrolyzates have also been found to inhibit dipeptidyl peptidase IV and to exert antioxidant properties, making them a promising functional ingredient with serum glucose lowering properties [33••]. However, the sequences of bioactive peptides have not been identified yet.

Although many quinoa components have been described to contribute on the beneficial effects on human health, to date, the evidence of these benefits demonstrated in both animals and humans is still limited. In a study inquiring the effects on lipid profile and glucose levels in male Wistar rats fed a fructose-enriched diet, it was demonstrated the ability of quinoa seeds to reduce serum total cholesterol, low density lipoproteins (LDL), triglycerides and glucose levels. Also, quinoa added to the diet was shown to inhibit the negative effects of fructose on high density lipoproteins (HDL) levels [34]. In another study, quinoa supplementation in diet administered to oxidative stress-induced rats reduced malondialdehyde levels in plasma and increased antioxidant enzymes activities [35]. These results indicate that quinoa seeds can protect animals from oxidative status by increasing their antioxidant capacity and reducing lipid peroxidation in plasma and different tissues. Foucault and co-workers investigated the potential role of quinoa to prevent diet-induced obesity in mice. Administration of 20HE-enriched quinoa extract to animals fed a high fat diet for 3 weeks resulted in the reduction of the development of adipose tissue in mice without changes in body weight gain. This adipose tissue-specific effect was associated to the down-regulation of expression of genes involved in lipid storage [36]. Few human trials have been conducted to evaluate the benefits of quinoa consumption (Table 2). Administration, twice a day, of 100 g quinoa in quinoa-added baby foods to 50–65 month old boys in low-income families in Ecuador for 15 days significantly augmented the plasma insulin-like growth factor (IGF-1) levels, when compared to the control group. Thus, it was indicated that baby food with quinoa provided sufficient protein and other essential nutritional elements capable to prevent malnutrition among kids [37]. Moreover, supplementation of diet with quinoa has been demonstrated to prevent cardiovascular disorders in healthy people [38] as well as to modulate metabolic parameters in postmenopausal overweight women [39•]. Quinoa was administered to celiac patients in order to evaluate the safety of its consumption as a gluten-free alternative to cereal grains [40•]. This study found, after 6 weeks consumption of 50 g quinoa daily, an improvement in gastrointestinal parameters and small decreases in total cholesterol, LDL, HDL and triglycerides levels.

Conclusions

Quinoa is a pseudocereal with an important tradition and notable environmental tolerance, in addition to its high nutritional value. It has been recently reported that one

serving of quinoa (about 40 g) meets a significant part of daily recommendations (RDA) for essential nutrients, mainly vitamins, minerals and essential amino acids. Moreover, a plethora of bioactives have been identified in this crop including saponins, phenolic compounds, phytosterols, phytoecdysteroids, and bioactive peptides. These compounds exert positive effects on different body systems helping to promote human health and to reduce risk of different chronic disorders. However, to date, few data demonstrating these health benefits are available, thus further research, including additional human clinical trials, would be needed to understand the biological properties of quinoa emphasizing on the phytochemicals' bioavailability, mechanisms of action, and interactions.

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Article demonstrating the safety of quinoa consumption by celiac patients as well as its health benefits.

Chapter

ANTIMICROBIAL AND ANTIOXIDANT PEPTIDES OBTAINED FROM FOOD PROTEINS

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ABSTRACT

Fruits, vegetables, oil seeds, nuts, cereals, spices, herbs, and grains are good sources of antioxidants such as phenolics, flavonoids and carotenoids. Research has been conducted on their antioxidant properties with in vivo and in vitro studies on extraction and purification of these components. Food habits are considered factors associated to health conditions. Presently, consumption of new functional food has increased, as the number of diseases related to nutritional habits has also increased. Consumers are interested in the potential benefits of nutritional support related to the different disease control or prevention. Functional food is known to play an important role in reducing health risks and stimulating health quality. The capacity of some plant-derived foods are known to reduce the risk of chronical diseases. The occurrence of secondary metabolites has been shown to exert a wide range of biological activities. In general, these metabolites have low potency as bioactive compounds when compared to pharmaceutical drugs. However, as they are ingested regularly and in significant amounts as part of the diet, they may have noticeable long-term physiological effects.

Food proteins have biological activities such as antimicrobial and antioxidant activities. Lactoferrin and lysozyme are two food proteins with potent antimicrobial activity. Food proteins can produce different peptides after hydrolysis with proteolytic enzymes. These peptides have different biological functions against the human organism. These peptides can have antimicrobial, antioxidant, antitumoral and anti-inflammatory activities. Bioactive peptides have between 3-20 amino acid residues; their bioactivity depends on the sequence and amino acid compositions. The antimicrobial and antioxidant peptides generated from food proteins are being studied in depth. These peptides are safer and healthier than synthetic drugs. Antimicrobial and antioxidant peptides contain between 5-16 amino acid residues and have a cationic charge.

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Keywords: proteins, bioactive peptides, antimicrobial activity, antioxidant activity, amino acids, lysozyme, lactoferrin, milk proteins, egg proteins

Introduction

It is presently well documented the value of proteins as an essential source of amino acids playing an important role in the human diet. It has also been recognized that dietary proteins have many other in vivo functionalities due to the biologically active peptides. These peptides are inactive within the sequence of the parent protein, but those peptides can be released by proteolytic enzymes during the gastrointestinal digestion (in vitro/in vivo methods) or by fermentation or ripening during food processing [1, 2].

Plant and animal food proteins are a source of a big number of bioactive peptides. These peptides have different uses within the pharmaceutical, medical, cosmetic, and food industries. In the science literature, peptides from food proteins are reported to have biological activities such as antimicrobial, antioxidant, antitumoral, anti-inflammatory, antiviral, opiate, antithrombotic, anti-hypertensive, immunomodulation or as relative to mineral utilization. Cholesterol-lowering properties have been reported during the last two decades. Bioactive peptides are small peptides composed of between 3 to 20 amino acid residues. There are also bioactive peptides with more amino acids such as Lunasin which is a food derived peptide from soybean with anticancer activity, composed of 43 amino acids. This peptide has also been found in amaranth seeds. A peptide's biological activity depends on their amino acids composition. These peptides are active when released from the precursor protein where they are encrypted inside the pattern protein. Once these peptides are available, they play a function in the different systems of the human body and produce positive effects in human health. Bioactive peptides can help reducing the risk of many chronic diseases such as hypertension, cardiovascular diseases and cancer [3, 4, 5].

Mellander in 1950 was the first to report bioactive peptides when he suggested that caseinophosphopeptides (casein derived phosphorylated peptides) enhanced the vitamin D activity in bone calcification in rachitic infants [6, 7] (Korhonen and Pihlanto 2003a).

Milk and egg proteins are the most important source of bioactive peptides with a great amount of research evaluating the different bioactivities of milk and eggs. Milk and egg peptides have the ability to promote benefits in human health and are considered good candidates in the formation of new products in the food industry. In the science literature, there is extensive research about the different peptide activities such as antimicrobial, antioxidant, anticancer, antihypertensive and other activities. Peptides have been identified by means of in vitro enzymatic digestion with different proteolytic enzymes. Other peptides found are obtained by modifying the synthetic sequence designed by software informatics [8, 9].

In the food industry, there are two biological activities of great importance, those being the antimicrobial and antioxidant activities. These activities are related to food security as microorganisms produce important economic losses in the world. Pathogenic microorganisms can produce intoxication and death when the toxins are ingested even in low doses. Due to this reason, it is important to find new natural molecules with the capacity of inhibiting and/or killing microorganisms such as bacteria. Lipid oxidation constitutes an important event in the conservation of processed foods such as oils, snacks, chips, and dry nut derivatives [10-12].

ANTIBACTERIAL ACTIVITY: FOOD PROTEINS AND PEPTIDES

Plants and animals produce special substances for protection against pathogenic microorganisms, many of these substances are bioactive peptides with antimicrobial capacity named antimicrobial peptides (AMPs). Presently, there are issues related to the growing number of pathogenic bacteria with high resistance to existent antibiotics for medical purposes, resulting in a big concern of public health in the world. For this reason, recent research has aimed to find a new molecule with antimicrobial activity. Peptides from food protein obtained from plants and animals are good candidates being that they are active and safe, have a low toxicity and are an economic production compared to the production of synthetic antibiotics [13, 14].

Antimicrobial peptides from plant and animal food proteins have been reported in the science literature. AMPs play a valuable role in the development of many organisms as well as being an important component of innate immunity response. Antimicrobial function of innate immunity is regulated by small peptides with a cationic charge (+2 to +9) such as lysine or arginine with the ability to kill Gram-negative bacteria, Gram-positive bacteria, fungi, and parasites as well as many viruses. The killing mechanism of the pathogenic microorganisms has been described as the perturbation to the integrity of the microbial cell membrane. However, other mechanisms have not been studied yet. More than 2,000 AMPs have been reported on line in different databases. Most of them are cationic peptides, and only a few of them are anionic, which share the ability to fold into amphipathic conformation upon interacting with the cell membranes in the microorganism [15].

The most important human antimicrobial peptides named defensins and cathelicidin are present in leukocytes and are also secreted by various epithelia in skin and mucosal surfaces including the ocular surface. AMPs can be classified into four groups based on their structures: α-helical peptides, β-sheet peptides, extended peptides, and loop peptides [16]. A-helical AMPs, including magainin, cecropin, and pexiganan, constitute a representative class of AMPs that are the most well established in structure-activity relationships.

The β-sheet AMPs, such as α-, β-defensins, and protegrin, are stabilized by disulfide bridges, and form relatively rigid structures. Many of β-sheet AMPs exert their antimicrobial activities by disrupting bacterial membranes. They are perpendicularly inserted or tilted into the lipid bilayer to form toroidal pores. Hydrophilic regions of the peptides are associated with the polar head groups of the membranes [17].

Extended AMPs, which are predominantly rich in specific amino acids such as proline, tryptophan, arginine, and histidine, have no regular secondary structure elements. Indolicidin is a tryptophan/proline-rich extended peptide and Bac5 and Bac7 are proline/arginine-rich peptides [18, 19].

Many extended AMPs are not active against the membranes of pathogens, but they can show their antimicrobial activities by way of penetrating across the membranes and interacting with the bacterial proteins inside [20]. Some extended peptides, such as indolicidin, are membrane active and induce membrane leakage. Indolicidin is a short 13-amino acid AMP containing five tryptophan and three proline residues. This peptide adopts helical structure in the presence of liposomes, and the high content of tryptophan residues interacts with lipid of membranes [18].

AMPs have a net positive charge (cationic) including in their structure amino acids such as Lys, Arg and His, and a high ratio of hydrophobic residues [19]. This positive charge allows the AMPs to selectively bind to negatively charged microbial membranes [20] over the mammalian membranes (Russell et al. 2011) [21]. Thus, the integrity of the bacterial cytoplasmic membranes barrier becomes compromised following initial AMP adsorption (Figure 1). A specific interaction of the AMPs with the bacterial membrane culminates in the loss of the cell contents and results in bacterial death. Although this interaction is most likely essential for the antimicrobial activity of AMPs, other mechanisms have been suggested. Among them, we can include the formation of pores by the

“barrel stave pore” or the “toroidal-pore” mechanisms, as well as the “carpet” mechanisms, based on detergent-like binding properties, highlighted in Figure 1 [22].

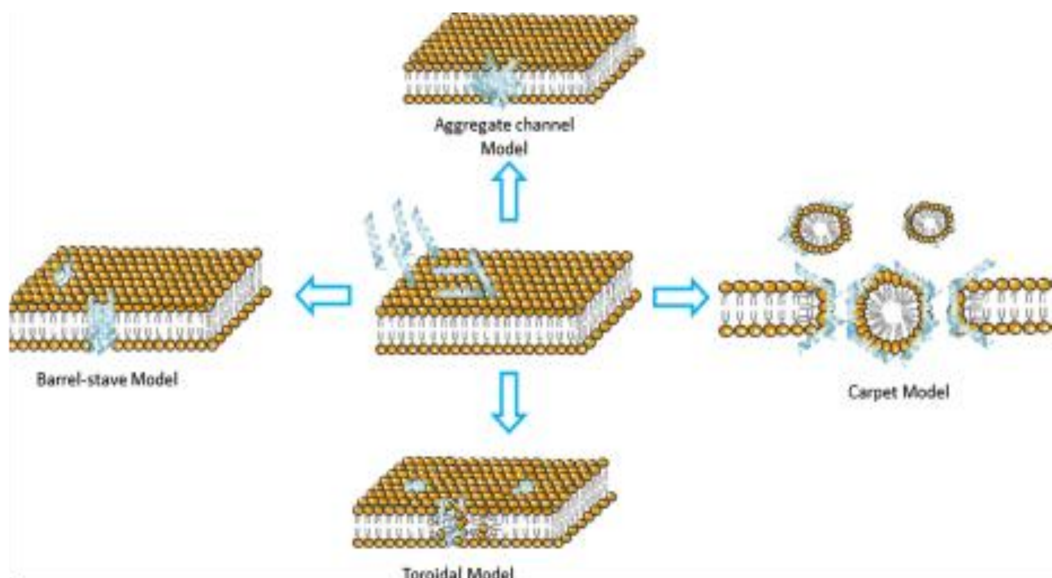

Figure 1. Bacterial membrane disruptions mechanisms following initial adsorption of AMPs. Pinto da Costa et al. (2015) [22].

Table 1. Bioactive peptides approved by FDA

Name of peptides	Source	Company
Pexiganan (MSI-78)	Frog (Xenopus laevis)	Magainin Pharmaceutical (USA)
Iseganan (IB-367)	Synthetic peptide, pig leucocytes	Intrabiotics Pharmaceuticals (USA)
Omiganen (MBI-226)	Synthetic peptide Bovine neutrophils	Microbiologix Biotech, Canada
MBI 594AN	Synthetic peptide Bovine neutrophils	Microbiologix Biotech, Canada
XMP.629	Synthetic peptide	Xoma (US) LLC USA
hLF1-11	Human	AM-Pharma, The Nederland
Neuprex (rBPI21)	Synthetic peptide	Xoma (US) LLC USA
Prialt®	*Conus magus*	Elan Pharmaceuticals
Pramlintide	*B Cells*	BMI
Exenatide	*Heloderma suspectum*	BMI
Lucinactant	Synthetic peptide	Discovery Labs

Presently, the finding of new antibacterial peptides is being studied. Although, the potential pool of thousands of natural peptides and millions of synthetic peptide possibilities is large, relatively few peptides have actually been accepted into clinical trials with a high number of patients. Antibacterial peptides were developed for clinical applications as pharmaceutical drugs to treat different diseases caused by bacteria. The most important success is that the FDA (Food and Drug Administration) accepted these molecules to be tested in humans using clinical trials. Many of these peptides were not accepted, however, other peptides are presently in phase II of assay. This situation opens the

possibility of testing new molecules to be used as antibiotics in the future. Table 1 shows different antibacterial peptides with clinical trial studies to be used as pharmaceutical drugs [23-25].

ANTIBACTERIAL PEPTIDES FROM MILK PROTEINS

Milk proteins are the best researched precursors of bioactive peptides [26, 27]. Casein and whey proteins are rich in good sequences exhibiting antihypertensive, opioid, antibacterial, antioxidant, antiviral and immunomodulating activities. Proteases are naturally occurring in food products, such as milk plasmin or hydrolyse proteins, and release bioactive fragments during processing or storage. Many types of bacteria applied in the production of fermented food and those occurring naturally in the gastrointestinal tract are capable of producing bioactive peptides. Moreover, they can be obtained in in vitro conditions of digestion with the help of proteases obtained from animals or bacteria.

Hill et al., 1874 identified the first peptide with antibacterial activity of αS_1 Bovine casein and named it Isradicine corresponding to the fragment f(1-23) of the N-terminal αS_1 Bovine casein. This antibacterial peptide was obtained using the chymosin enzyme. Isradicine presents antibacterial activity against *Lactobacillus* and Gram-positive bacteria [28].

αS_2-caseína bovina was used to obtain two peptides with hydrolysis in vitro with pepsin, these peptides were identified as f(183-207) and f(164-179). They were able to inhibit *Escherichia coli* ATCC 25922, *Escherichia coli* MC 1061, *Listeria innocua, Bacillus cereus P7* and *Streptococcus thermophiles* [29].

Glyco-macropeptide (GMP) and caseinomacropeptide (CMP) are formed after a specific cleavage of bovine casein by the chymosin enzyme. Caseinomacropeptide (CMP) may exhibit an inhibitory activity against *Staphylococcus mutans* and *Escherichia coli* whereas GMP modulates the gut microflora [30].

Lactoferrin and Its Antibacterial Activity

Milk contains a wide variety of proteins that hold important biological functions, in addition to their nutritive value. These active proteins are mainly present in the whey fraction and some of them have a defensive role, such as immunoglobulins, lactoferrin, lactoperoxidase and lysozyme. Lactoferrin is an iron-binding glycoprotein of the transferrin family that plays a protective function in the innate immune response. It is produced by the epithelial cells of the mammary gland and secreted into milk. Lactoferrin can also be found in several mucosal secretions like tears, saliva, gastrointestinal fluids, urine and seminal fluid, and in the secondary granules of neutrophils, being released in locations where there is an inflammatory response. LF content in milk varies depending on the species. The amount of LF is lower in cows' milk (0.1–0.4 mg mL^{-1} than in human milk (1–3 mg mL^{-1}). However, a factory scale technology to produce large amounts of bovine LF (bLF) at high purity from cows' milk was established over 20 years ago [31].

Lactoferrin consists of a single-polypeptide chain of approximately 80 kDa composed of two lobes, each of which contains a binding site for a ferric iron. LF is a major component of milk and is present in neutrophil granules or other exocrine secretions such as tears, saliva, and the cervical mucus. LF is thought to play a role in innate defense and exhibits a diverse range of biological activities, including antimicrobial activities, antiviral activities, antioxidant activities, immunomodulation, modulation of cell growth, and binding and inhibition of several bioactive compounds, such as lipopolysaccharide and glycosaminoglycan. Due to this iron-binding capacity, it can exert bacteriostatic and bactericidal effects against a wide range of Gram-positive and Gram-

negative bacteria, since iron is an essential nutrient for bacterial growth. We found that pepsin-hydrolysate of LF (LFhyd) has more potent antimicrobial activity than native protein and we purified the active peptide from LFhyd [32]. The antimicrobial peptide derived from LFhyd was named lactoferricin [33]. Interestingly, LFcin and its derivatives exhibit various biological activities, like LF. Therefore, the LFcin-region seems likely to be an important functional domain of LF.

ANTIBACTERIAL PEPTIDES FROM EGG PROTEINS

Egg proteins are nutritionally complete with a good balance of essential amino acids which are needed for building and repairing the cells in muscles and other body tissues [34]. Egg proteins are distributed in all parts of eggs, but most of them are present in egg white and egg yolk amounting to 50% and 40%, respectively. The remaining amount of protein is in egg shell and egg shell membranes. Different antimicrobial peptides obtained from egg proteins have been reported. OTAP-2 is an antimicrobial peptide obtained from ovotransferrin protein with a strong antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* [35].

Antimicrobial Activity of Hen Egg Lysozyme (HEL)

Lysozyme (E.C.3.2.17, N-acetyl-muramic-hydrolase), is a globular basic protein found in nature and is characterized by its high enzymatic activity. It was first discovered in nasal mucous, from a patient with coryza, by Alexander Fleming who named it 'Lysozyme' as he observed its lytic activity towards bacterial cocci [36]. He later found that many regions within the human body contain lysozyme and it is now known to be present in almost all secretions, body fluids, and tissues of human and animal organisms; with tears, nasal secretions and sputum being the richest sources. It has also been isolated from some plants, bacteria, and bacteriophages [37]. Within the animal kingdom, three major types of lysozyme have been reported, usually designated as the chicken type (c-type), the goose type (g-type) and the invertebrate type (i-type). They have different amino acid sequences along with differing biochemical and enzymatic properties. The archetype of lysozyme, being the c-type, which is commonly found in hen egg, has been widely used as a research model for studies concerning enzyme structure and function [38]. Egg albumen is known to have an exceptionally high amount of lysozyme, normally referred to as hen´s egg lysozyme (HEL), representing 3.4% of the egg white protein content [39]. Its main physicochemical features are summarized in Table 2. The complete amino acid sequence of HEL, being unique with respect to other types of lysozyme, was deduced in 1963 after a series of enzymatic digestion experiments [40]. Found to be a single polypeptide chain consisting of 129 amino acids with a Lys placed at the N-end amino acid and a Leu at the C-end one. In addition, HEL contains four disulfide bridges, which enable the enzyme to have a high thermal stability, along with six helix regions [41]. The molecule has a molar mass of 14.3 kDa and is ellipsoidal in form with the dimensions of about 45 X 30 X 30 Å, as was first observed by Blake et al. [42] through research using X-ray analysis procedures.

HEL has been associated with many biological activities such as antibacterial, antiviral, immunomodulating, immunostimulating and antitumoral among others [43, 44]. In fact, it is thought that the hen's egg has a high lysozyme content in order to function as protection of the developing embryo, specifically since it is seen to be connected with the degradation of Gram positive bacteria cell walls, inactivation of viruses, and inactivation of toxins outside of cells [45]. Due to its strong antibacterial and antimicrobial effects, HEL has become a valuable substance widely used in food preservation, cosmetics, and pharmaceutics through a bioprotective or natural antibiotic manner [46]. There are various methods for separating lysozyme from egg white, for the use in the industry as a low cost product ingredient, including chromatographic and ultrafiltration techniques. HEL has a long history for being thought of as a safe and natural food component, and additional intake as a

preservative was not considered to be a hazard to human health. It has been generally recognized as safe (GRAS) status by the World Health Organization (WHO) and the Food and Drug Administration (FDA) in the year 2000 [47]. Unfortunately, now HEL is also known for its allergenic potential [48]. In this regard, people with egg allergy may be allergic specifically to HEL, and this poses a major problem for them since HEL can be found in many food products and drugs [49].

Table 2. Physicochemical properties of HEL

Hen Egg White Lysozyme		
Molecular weight	14.3 kDa	
Subunits	1	
Length	129 amino acids	
Amino acid residues	Alanine (12) Arginine (11) Aspartic acid (21) Cysteine (8) Glycine (12) Glutamic acid (5) Histidine (1) Isoleucine (6) Leucine (8)	Lysine (6) Methionine (2) Phenylalanine (3) Proline (2) Serine (10) Threonine (7) Tryptophan (6) Tyrosine (3) Valine (6)
Positive amino acid	18 positive amino acid: Arg, Lys, His	
N-terminal amino acid	Lysine	
C-terminal amino acid	Leucine	
Isoelectric point	10.7-11	
Disulfide bonds	4	
Charge at pH 7.0	positive	

HEL belongs to a class of enzymes that lyse the cell wall of certain Gram-positive bacteria by splitting β-1-4-linkages of the glycosidic bond between N-acetyl muramic acid and N-acetylglucosamine of the peptidoglycan [50], the polymer which makes up bacterial cell walls (Figure 2). The active site of HEL is formed by six sub-sites, which can bind six sugar residues, and cleft position including the catalytic groups Glu-35 and Asp-52. Gram-positive bacteria are more prone to disruption by HEL action since their cell wall contains around 90% of peptidoglycan, whereas Gram-negative bacteria contain only 5-10% peptidoglycan, and this peptidoglycan lies beneath the outer membrane of the cell envelope. The lipopolysaccharide layer of the outer membrane functions as a shield against macromolecules and hydrophobic compounds, making it more stable to the action of HEL [51]. Therefore, HEL is the most effective against Gram-positive bacteria such us *Bacillus stearothermophilus*, *Clostridium tyrobutyricum*, *Clostridium thermos-saccharolyticum, Clostridium sporogenes* and *Bacillus* spp. while it is less effective against Gram-negative bacteria because of these structural differences [52].

The antibacterial properties of HEL have been the focus of many studies. Several authors have proposed that the antibacterial properties of HEL are not dependent on its muramidase activity because some denatured or partially denatured and catalytically inactive forms of HEL have shown to keep or even enhance their antimicrobial activity [53, 54, 55], which could be due to the conformational changes leading to increased hydrophobicity. Moreover, a catalytically inactive mutant of HEL showed the same antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* as that of the wild type of HEL demonstrating that the antimicrobial activity of HEL is operationally independent of its muramidase activity [56]. On the contrary, other studies conducted on different Gram-negative bacteria under high hydrostatic pressure support the importance of the

enzymatic activity of HEL in regards to its antimicrobial activity [57]. Therefore, the antimicrobial activity of HEL might be a combination of the structural properties of HEL and its direct bacteriolytic action depending on the nature of the bacteria.

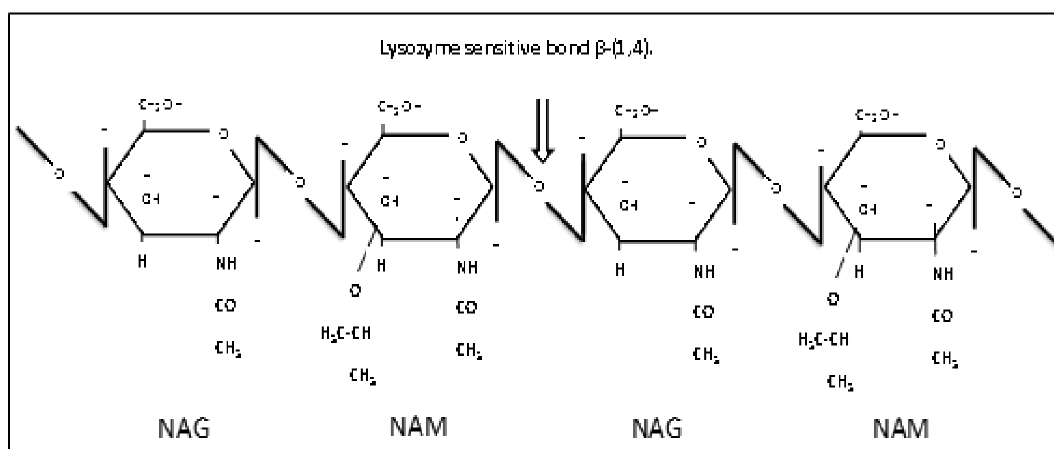

Figure 2. Structure of a repeating unit of the peptidoglycan cell wall structure showing the cleavage site. NAG stands for N-acetyl muramic acid and NAM stands for N-acetylglucosamine.

Several strategies have been developed to extend the working spectrum of HEL against Gram-negative bacteria and/or increase its efficacy on Gram-positive bacteria. These include partial or complete denaturation of HEL by heating and/or chemical treatments [58, 59] as well as HEL polymerization [25]; pre-treatment of Gram-negative bacteria with chelating agents, such as ethylenediaminetetraacetic acid (EDTA) for sensitizing them to HEL´s action [26]; attachment of various fatty acids (caproic, capric, myristic, palmitic) for enhancing HEL´s interaction with cell membrane [60, 61, 62]; conjugation of HEL with perillaldehyde, a major phenolic aldehyde in the steam distillate of the green leaves of Japanese shiso (Perilla frutecens) [63]; binding of hydrophobic peptides to HEL at its C-terminus by genetic modification [64], or the synergistic use of HEL together with other natural preservatives, i.e., nisin, lactoferrin, glycin, organic acids, aprotinin and gelatin among others [65]. Furthermore, glycosylation of HEL with different carbohydrates (dextran, galactomannan, xyloglucan, etc.) through Maillard reaction could lead to a glycosylated HEL with enhanced or unmodified antimicrobial activity but having improved functional properties [66, 67, 68], which would be of great interest for food applications. For example, recently a procedure has been optimized for HEL glycation with galactose, galactooligosaccharides and potato galactan by the Maillard reaction for the production of prebiotic glycoproteins [69].

Besides these possibilities, one of the most interesting approaches for maximizing the use of HEL in the food and pharmaceutical industry is the use of specific HEL-derived-peptides with strong antimicrobial activity. It has been reported that HEL contains peptide sequences that can induce non catalytic bacterial death different from enzymatic lysis of cell membranes. The clostripain-digested HEL yielded an antimicrobial pentadecapeptide (amino acids 98-112) located at the domain called helix-loop-helix (amino acids 87-114), which is recognized as the antimicrobial active site of the protein [70], effective against both Gram-negative and Gram-positive bacteria [71]. The same study showed the importance of cationic and hydrophobic amino acids as the antimicrobial activity of the pentadecapeptide decreased significantly or even was lost when Trp 108 or Trp 111 were replaced with Tyr, while the opposite effect occurred when Asp 106 was replaced by Arg. In fact, it is known that some antimicrobial peptides are rich in Trp as it is in the case of tritrpcin or indolicidin, which are cationic peptides of 13 amino acids that likely destroy bacterial cells by forming ion channel-like structures in planar lipid bilayers [72]. Unlike pepsin or clostripain, other proteolytic enzymes such

as trypsin, chymotrypsin and papain do not hydrolyze HEL unless they are combined with other enzymes or HEL is denatured. For example, Mine et al. [73] digested HEL with pepsin and subsequently with trypsin obtaining two bacteriostatic peptides, one located in the middle part of the helix-loop-helix (residues 98-108) and the other corresponding to amino acid residues 15-21 of HEL that were active against Gram-negative (*Escherichia coli* K-12) and Gram-positive bacteria (*Staphylococcus aureus* 23-394) respectively. Moreover, a novel study from Memarpoor-Yazdi et al. [74] characterized a hydrolyzate from HEL digested with papain and trypsin, finding a peptide composed of 16 amino acids (residues 46-61). This peptide showed a strong antioxidant activity as well as antimicrobial effects on *Escherichia coli* and *Leuconostoc mesenteroides*, reinforcing the potential role of HEL as a source of bioactive peptides with practical applications in foods or drugs. Two peptides, RAWVAWR-NH2 and IVSDGNGMNAWVAWR-NH2, derived from human and chicken lysozyme, respectively, exhibit antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* [75]. Carrillo et al., 2014 reported modified lysozyme by heat and hydrolysis with antimicrobial activity against lactic bacteria [76].

Antioxidants Activity: Food Proteins and Peptides

Reactive oxygen species (ROS; superoxide, hydrogen peroxide, and hydroxyl radicals) and free radical-meditated reactions can cause oxidative damage to cellular structures and functional molecules (i.e., DNA, proteins, and lipids), and therefore lead to many diseases, such as aging, cancer, diabetes, cardiovascular disease, Alzheimer's disease, and other neurodegenerative disorders [77]. Antioxidants are thought to be highly effective in the management of ROS-mediated tissue impairments. Antioxidants are used worldwide as food additives to protect foodstuffs against deterioration caused by oxidation, such as fat rancidity and colour changes [78]. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are two phenolic antioxidants that are widely used as a synthetic food additive to preserve oils and fats. The use of BHA and BHT and their metabolites is not restricted to foodstuffs. Considering the potential adverse effects as cancerogenic of the synthetic antioxidants (BHA, BHT and TBHQ), natural antioxidants derived from dietary sources have received much attention in recent years [79, 80]. The use of protein hydrolysates or peptides to obtain the antioxidant capacity in functional foods present additional advantages over other natural antioxidants, since they also confer an additional nutritional value (proteins are essential nutrients), as well as other desired functional properties, e.g., good water solubility, emulsion and foaming properties [81, 82]. Peptides may be present in foods together with other antioxidants; therefore, it would be interesting to study the interactions of peptides and other nonpeptidic antioxidant agents, e.g., phytochemicals.

Bioactive peptides have between 2-20 amino acid residues; their bioactivity depends on the sequence and amino acid compositions [83, 84]. Recently, attention has mainly focused on the antioxidant peptides generated from food proteins. These peptides are safer and healthier than synthetic drugs [85]. Antioxidant peptides contain 5-16 amino acid residues. Their antioxidant activities can be related to ion chelating, radical scavenging and inhibition of lipid peroxidation. The importance of positively charged amino acids in determining the strength of peptides as antihypertensive and antioxidant has been indicated in different studies. Strong antimicrobial peptides are cationically charged. Those cationically charged peptides contain amino acids such as Lys, Arg and His [86, 87]. Lysozyme has an isoelectric point of pI=10.7, with a high content of positively charged amino acids. Moreover, antioxidant peptides often possess hydrophobic amino acid residues such as Pro, His, Tyr, Trp, Met, or Cys in their sequences and Val or Leu at the N-terminus [88]. Lysozyme may be a great substrate for production of bioactive peptides with antioxidant activity. You et al., (2010) [89] have described two antioxidant fractions of hydrolysate of lysozyme with pepsin containing positively charged amino acids such as f(13-20)KRHGLDNY, f(14-23)RHGLDNYRGY and f(13-23)RHGLDNYRGY. Moreover, many researchers have reported

that peptides and proteins hydrolyzed from various food sources have significant antioxidant activity [90]. Furthermore, hen egg white lysozyme suppresses reactive oxygen species (ROS) generation and protects against acute and chronic oxidant injuries [91, 92]. Many proteins have been shown to have antioxidative activities against the peroxidation of lipids or fatty acids upon hydrolysis (Tsuge et al., 1991) [93, 94]. Thus, the antioxidative activities either of amino acids or peptides have been investigated to gain insight into the antioxidative mechanism of protein hydrolysates. Several amino acids, such as Tyr, Met, His, Lys, and Trp, are generally accepted as antioxidants in spite of their pro-oxidative effects in some cases [95, 96].

Carrillo et al., 2016 [76] have reported five peptides from HEL with antioxidant activity [100]. Hen egg lysozyme was hydrolyzed with pepsin in situ on a cation-exchange column to isolate antioxidant peptides. The most cationic fraction was eluted with 1 M NaCl. Five positively charged peptides f(109-119) VAWRNRCKGTD, f(111-119) WRNRCKGTD, f(122-129) AWIRGCRL, f(123-129) WIRGCRL and f(124-129) IRGCRL were identified using tandem mass spectrometry. Using ORAC-FL, all five peptides presented antioxidant activity with values of (1,970; 3,123; 2,743; 2,393 and 0.313 μmol Trolox/μmol peptide) respectively. Using the TBARS method in the zebrafish larvae model, all five synthetic peptides were found to efficiently inhibit lipid peroxidation (36.8; 51.6; 55.56; 63.2; 61.0% inhibition of lipid peroxidation) respectively.

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