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Proteases Digestivas do Hepatopâncreas dos Camarões Marinhos *Farfantepenaeus subtilis* e *Farfantepenaeus paulensis*

DIEGO DE SOUZA BUARQUE

Prof. Dr. RANILSON DE SOUZA BEZERRA
Orientador

Prof. Dr. LUIZ BEZERRA DE CARVALHO JÚNIOR
Co-orientador

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DIEGO DE SOUZA BUARQUE

Proteases Digestivas do Hepatopâncreas dos Camarões Marinhos
Farfantepenaeus subtilis e *Farfantepenaeus paulensis*

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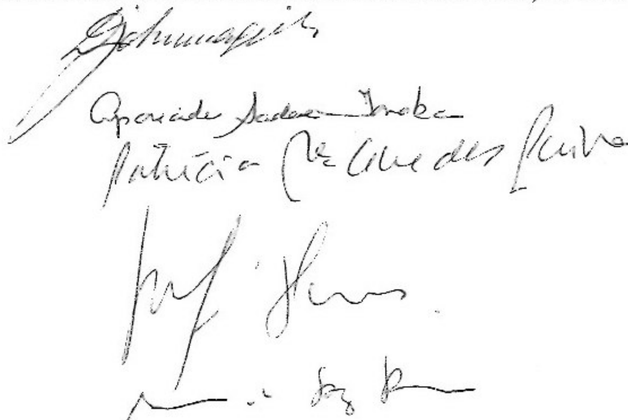
Ata da defesa de dissertação do Mestrando **Diego de Souza Buarque,** realizada em 21 de fevereiro de 2008, como requisito final para obtenção do título de Mestre em Bioquímica e Fisiologia da UFPE.

Às 14:40 horas, do dia vinte e um de fevereiro de 2008, foi aberto no Auditório Prof. Marcionilo Lins – Depto. De Bioquímica do Centro de Ciências Biológicas da Universidade Federal de Pernambuco, o ato de defesa de dissertação do mestrando **Diego de Souza Buarque**, aluno do Curso de Mestrado em Bioquímica e Fisiologia/CCB/UFPE. Iniciando os trabalhos a Profa. Dra. Patrícia Maria Guedes Paiva, na qualidade de Vice-Coordenadora do Curso acima citado, fez a apresentação do aluno, de seu orientador Prof. Dr. Ranilson de Souza Bezerra, de seu co-orientador Prof. Dr. Luiz Bezerra de Carvalho, bem como da Banca Examinadora composta pelos professores doutores: Ranilson de Souza Bezerra, na qualidade de Presidente, Patrícia Maria Guedes Paiva, ambos do Depto. de Bioquímica/UFPE, Aparecida Sadae Tanaka, da UNIFESP, e Daniel Eduardo Lavanholi de Lemos, do Dpto. de Oceanografia Biológica/USP. Após as apresentações, o Prof. Dr. Ranilson de Souza Bezerra convidou o aluno para a apresentação de sua dissertação intitulada: **"Proteases Digestivas do Hepatopâncreas dos Camarões Marinhos *Farfantepenaeus subtilis* e *Farfantepenaeus paulensis*",** e informou que de acordo com o Regimento Interno do Curso, o candidato dispõe de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pelo aluno para responder às perguntas será de 30 (trinta) minutos. O aluno procedeu à explanação e comentários acerca do tema em 35 **(trinta e cinco)** minutos. Após a apresentação do mestrando, o Sr. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra a primeira examinadora, Profa. Dra. Aparecida Sadae Tanaka, que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, a referida professora deu-se por satisfeita. Logo após, o Sr. Presidente passou a palavra para o Prof. Dr. Daniel Eduardo Lavanholi de Lemos, que agradeceu ao convite, fez alguns comentários e sugestões, iniciando sua argüição. Ao final, o referido professor deu-se por satisfeito. Em seguida, o Sr. Presidente passou a palavra para a. Profa. Dra. Patrícia Maria Guedes Paiva que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, a referida professora deu-se por satisfeita. Em seguida, o Sr. Presidente passou da palavra para o co-orientador que fez alguns comentários a respeito do trabalho do aluno. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovado com Distinção**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 21 de fevereiro de 2008.



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RESUMO

Os camarões *Farfantepenaeus subtilis* e *Farfantepenaeus paulensis* são espécies nativas e aparecem como uma alternativa ao cultivo do *Litopenaeus vannamei*, que hoje corresponde a mais de 90% do cultivo de camarões marinhos da região nordeste. Proteases do hepatopâncreas de *F. subtilis* jovens e adultos e *F. paulensis* jovens foram estudadas quanto aos seguintes aspectos: inibição enzimática, pH e temperatura ótima, estabilidade térmica, eletroforese e zimogramas. No extrato bruto de *F. subtilis*, não houve diferenças significativas nas atividades proteolíticas em ambas as fases de vida utilizando azocaseína, leucina p-nitroanilida (Leu-p-Nan) e substratos β-naftilamida ($p \geq 0{,}05$). No entanto, as atividades tríptica (Benzoil arginina p-nitroanilida-BApNA) e quimotríptica (Succinil alanina alanina prolina fenilalanina p-nitroanilida-SAPNA) foram mais elevadas em jovens do que em adultos ($p < 0{,}05$). Atividades de tripsina e quimotripsina também foram detectadas em *F. paulensis*. Tosil lisina clorometil cetona (TLCK) e benzamidina inibiram fortemente as atividades proteolíticas nos extratos de *F. subtilis* e *F. paulensis*. Tosil fenilalanina clorometil cetona (TPCK) foi capaz de inibir 59,34% da atividade de quimotripsina em *F. paulensis* utilizando SAPNA como substrato. A temperatura ótima para tripsina-símile e quimotripsina-símile em ambas as fases de vida foi 55°C, enquanto que para aminopeptidases houve uma diferença entre jovens (55°C) e adultos (45°C). Tripsina-símile reteve aproximadamente 15% de sua atividade inicial quando incubada a 55°C por 30 minutos, enquanto quimotripsina-símile e leucina aminopeptidase-símile retiveram 60% e 45% de atividade respectivamente. No extrato bruto de *F. paulensis* a tripsina-símile apresentou atividade máxima em pH 8,0 e temperatura ótima de 40°C. A atividade mais elevada de quimotripsina-símile foi detectada numa faixa de pH alcalino (7,2-9,0) e na temperatura de 55°C. O zimograma de estabilidade térmica apresentou um padrão similar de bandas com atividade proteolítica em *F. subtilis* jovens e adultos, exceto que o extrato de camarões juvenis apresentou uma banda termoestável a 65°C. PMSF inibiu todas as bandas proteolíticas. Duas isoformas de quimotripsina (31,6 e 36,4 kDa) foram estáveis a 85°C em *F. paulensis*. A caracterização de enzimas digestivas de camarões nativos pode gerar informações importantes para o entendimento da fisiologia e da capacidade digestiva destes organismos, principalmente pelo fato dessas enzimas estarem diretamente ligadas ao aproveitamento de aminoácidos dietários, sendo estes importantes fontes de monômeros para a síntese de proteínas funcionais que serão responsáveis por uma

série de eventos fisiológicos como: crescimento, reprodução, defesa imunológica, entre outros.

Palavras-chave: *Farfantepenaeus subtilis*, *Farfantepenaeus paulensis*, tripsina, aminopeptidases, quimotripsina.

ABSTRACT

Farfantepenaeus subtilis and *Farfantepenaeus paulensis* are native shrimp species and appear as an alternative to *Litopenaeus vannamei* cultivation, which represents more than 90% of the farmed shrimp in the Northeast of Brazil. Proteases from hepatopancreas of *F. subtilis* juveniles and adults and *F. paulensis* juveniles were studied according to the following properties: effect of inhibitors, optima pH and temperature, thermal stability, electrophoresis and zymograms. There are no significant differences on proteolytic activities between both life stages using azocasein, Leucine-p-nitroanilide (Leu-p-Nan) and aminoacyl-β-naphthylamide as substrates ($p \geq 0.05$). Trypsin-like (Benzoyl arginine p-nitroanilide - BapNA) and chymotrypsin-like (Succynil alanine alanine proline phenilalanine p-nitroanilide – SAPNA) activities were higher in *F. subtilis* juveniles than adults extracts ($p < 0.05$). Trypsin-like and chymotrypsin-like activities were observed in crude extract of *F. paulensis.* Tosyl-lysine chloromethyl ketone (TLCK) and benzamidine inhibited proteolytic activity in *F. subtilis* and *F. paulensis*. Tosyl phenylalanine chloromethyl ketone (TPCK) was able to inhibit 59.34% of chymotryptic activity using SAPNA as substrate. The optimum temperature for trypsin-like and chymotrypsin-like activities for both life stages was 55ºC, while for aminopeptidase activity there are a difference between juveniles (55ºC) and adults (45ºC). Trypsin retained about 15% of the initial activity when incubated at 55ºC for 30 min while chymotrypsin-like and leucine aminopeptidase-like retained 60% and 45% of activity respectively. The optimum pH for trypsin-like was 8.0 and activity was maximum at 40°C on crude extract of *F. paulensis*. The highest chymotrypsin-like activity was obtained at alkaline pH range (7.2-9.0) at 55°C. The thermal stability zymogram of crude extract of *F. subtilis* showed a similar proteolytic band pattern between juvenile and adult shrimps, except that juvenile shrimps presented one proteolytic thermostable band at 65ºC. PMSF strongly inhibited all proteolytic bands. Two possible thermal resistant (85°C) chymotrypsin isoforms (31.6 and 36.4 kDa) were found in *F. paulensis*. The characterization of digestive enzymes from native shrimps can provide relevant informations for understanding the physiology an the digestive abilities of these organisms. These enzymes are responsible for breaking down dietary proteins into aminoacids which are source of impotant monomers used for the synthesis proteins related to physiological events such as growth, reproduction and immunological response.

Keywords: *Farfantepenaeus subtilis*, *Farfantepenaeus paulensis*, trypsin, aminopeptidases, chymotrypsin.

1 - INTRODUÇÃO

1.1 Carcinicultura Marinha no Brasil

O cultivo de camarões marinhos no Brasil teve início na década de 70, na Região Nordeste, com a introdução da espécie exótica *Marsupenaeus japonicus* (Barbieri Junior & Ostrensky Neto, 2002).

Porém, apesar de *M. japonicus* ser uma das espécies mais importantes cultivadas na Ásia, ela não se adaptou bem às condições brasileiras, o que fez com que os produtores tentassem utilizar espécies nativas em suas fazendas, porém, novamente sem sucesso (Barbieri Junior & Ostrensky Neto, 2002).

No início dos anos 90, a introdução da espécie *Litopenaeus vannamei* revolucionou a carcinicultura marinha no Brasil (Barbieri Junior & Ostrensky Neto, 2002). A partir dessa década, o cultivo de camarões se tornou uma atividade importante e bastante rentável (Burgos-Hernández *et al.*, 2005). Em pouco tempo, *L. vannamei* demonstrou uma adaptação bastante satisfatória, apresentando níveis de produtividade e competitividade muito superiores aos até então alcançados com as espécies cultivadas no Brasil (Barbieri Junior & Ostrensky Neto, 2002). Por isso, atualmente esta espécie corresponde a mais de 90% do cultivo de camarões marinhos no nordeste.

A evolução na produção de camarões obtida com a introdução de *L. vannamei* no país pode ser expressa em números: em 1994, o Brasil produzia 2.385 t de camarões; em 2001, a produção já ultrapassava 38.000 t, um aumento de quase 1.500% (Barbieri Junior & Ostrensky Neto, 2002). De acordo com dados da Associação Brasileira dos Criadores de Camarão (ABCC), em 2003, o Brasil já ocupava o sexto lugar entre os maiores produtores mundiais e o que é mais importante, com o maior nível de produtividade por área cultivada, cerca de 6.084 Kg/ha/ano. A produção total passou de 3.600 toneladas em 1997, com uma área cultivada de 3.548 hectares, para 90.190 toneladas em 2003, em uma área de 14.824 hectares (Rocha & Rodrigues, 2003). Na Tabela 1 pode ser visto o perfil da evolução da carcinicultura marinha brasileira nos anos de 2000 e 2002.

Tabela 1: Perfil da Carcinicultura Brasileira nos anos de 2000 e 2002.

Discriminação	2000	2002
Área de operação	7.800 ha	11.016 ha
Produção de camarões	30.000 t	60.128 t
Produtividade média	3.846 Kg/ha/ano	5.458 Kg/ha/ano
Nº de laboratórios em atividade	20	28
Produção de pós-larvas	6 bilhões	11,4 bilhões

Fonte: ABCC - Associação Brasileira de Criadores de Camarão e Rocha & Rodrigues, 2003.

Em 2004, houve uma retração na atividade em relação à produção total, que atingiu 74.904 t e os níveis de produtividade diminuíram para 4.510 kg/ha/ano, tomando-se como base o ano anterior (Rodrigues, 2005). Em 2007 o declínio da carcinicultura foi ainda mais acentuado, tomando como base os dados de produção (65.000 t) e produtividade (4.063 Kg/ha/ano) (Rocha, 2007a). As causas apontadas para justificar esse declínio dizem respeito a condições climáticas e, principalmente, à ocorrência da enfermidade viral, denominada de Mionecrose Infecciosa (IMN) em fazendas de cultivo instaladas, principalmente, nas áreas costeiras dos estados do Piauí, Ceará e Rio Grande do Norte (Rodrigues, 2005). O desempenho da atividade entre os anos de 2003 e 2007 está demonstrado na Tabela 2.

Tabela 2: Desempenho da carcinicultura marinha brasileira em 2003 e 2007.

Variáveis levantadas/ano	2003	2007	Variação (%)
Área (ha)	14.824	16.000	7,9
Produção (ton)	90.190	65.000	-27,9
Produtividade (Kg/ha/ano)	6.084	4.063	-33,2

Fonte: Rocha, 2007a.

Na fase de maior desenvolvimento, a atividade desempenhava um papel de extrema relevância, principalmente, no que diz respeito à geração de renda e divisas e à criação de empregos no meio rural. No primeiro caso, a região Nordeste tinha no camarão cultivado um dos mais importantes produtos de exportação do setor primário, tendo alcançado em 2003, um volume de 90.190 toneladas correspondente a um valor total de US$ 226 milhões. Quanto ao aspecto social, a atividade chegou a gerar 3,75 empregos diretos e indiretos por hectare de produção, devendo-se salientar que quase 80% da mão-de-obra empregada não têm qualificação (Sampaio & Costa, 2003).

1.2 *Farfantepenaeus subtilis* e *Farfantepenaeus paulensis*

Embora as técnicas de cultivo para *L. vannamei* sejam de domínio satisfatório, a ocorrência de enfermidades provocadas por vírus, responsável pela queda drástica na produção de diversos países, levou o governo brasileiro a tomar medidas para proibir a entrada de crustáceos no país. A indústria nacional do camarão marinho cultivado teve de conviver com a impossibilidade de importação de novas matrizes e com a ameaça de diminuição da variabilidade genética da população existente no Brasil, o que poderia provocar não só uma redução nas taxas de crescimento obtidas, como tornar o *L. vannamei* uma espécie ainda mais susceptível ao ataque de doenças. Atualmente, a ocorrência de doenças juntamente com a queda do câmbio e a ação antidumping também geram dificuldades para que o cultivo desta espécie volte a ser rentável (Rocha, 2007b).

Este fato tem gerado uma grande mobilização da comunidade científica para desenvolver pesquisas com camarões marinhos nativos, como as espécies *Farfantepenaeus subtilis* e *Farfantepenaeus paulensis*, pois a procura de espécies alternativas contribui, em muitos casos, para uma expansão sustentável da carcinicultura, bem como para uma maior diversificação no comércio (Lemos *et al.*, 2002). Porém a falta de informações sobre a biologia de espécies nativas e suas relações ecológicas dificulta o seu cultivo e a sua comercialização.

Assim como o *L. vannamei*, as espécies *F. subtilis* e *F. paulensis* pertencem à família Penaeidae, sendo diferenciadas da primeira apenas em gênero e espécie (Tabela 3).

Tabela 3: Identificação taxonômica de *F. subtilis* e *F. paulensis*.

Táxons	Classificação
Filo	Arthropoda
Sub-filo	Crustácea
Classe	Malacostraca
Subclasse	Eumalacostraca
Superordem	Eucarida
Ordem	Decapoda
Subordem	Dendrobranchiata
Superfamília	Penaeoidea
Família	Penaeidae
Gênero	*Farfantepenaeus*
Espécie	*F. subtilis* / *F. paulensis*

Modificada de Barbieri Junior & Ostrensky Neto, 2001.

Camarões peneídeos, os quais estão entre os mais importantes camarões cultivados (Shiau, 1998), apresentam várias semelhanças morfológicas, de forma que é possível apresentar uma estrutura geral para os camarões pertencentes a esta família (Figura 1).

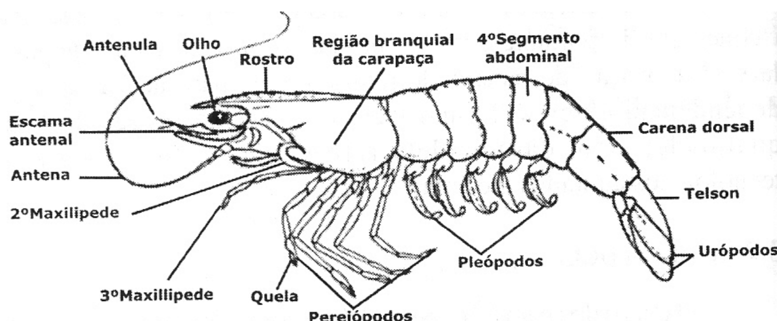

Figura 1: Morfologia de camarões da família Penaeidae. Barbieri Junior & Ostrensky Neto, 2002.

Porém, as maiores diferenças entre estas espécies nativas e *L. vannamei* podem estar a nível fisiológico, já que, segundo Nunes *et al.* (1997), *F. subtilis* apresenta uma maior tendência a se alimentar de proteína animal do que *L. vannamei*, o qual apresenta um hábito alimentar onívoro. Com isso, a ração deve ser diferente para estas espécies, já que elas apresentam diferentes requerimentos nutricionais, além de necessitarem dos compostos em quantidades diferentes. Em experimentos realizados pela iniciativa privada com *F. subtilis*, foram obtidos excelentes resultados no que se refere ao crescimento, entretanto, as taxas de conversão alimentar mostraram-se altas (Maia & Nunes, 2003).

1.3 Enzimas Digestivas

Os animais relacionam-se com o meio ambiente através de vários estímulos, podendo-se destacar o alimento como um dos mais importantes. Através deste, heterótrofos obtêm a energia necessária para sintetizar moléculas requeridas para o desenvolvimento, sobrevivência e realizar ações tais como: os movimentos, reprodução e defesa. Para utilizar esta fonte de energia, o alimento é submetido à ação de enzimas, as quais contribuem para a quebra das principais macromoléculas em compostos que podem ser absorvidos (Córdova-Murueta *et al.*, 2003). A digestão tem sido uma área razoavelmente estudada em relação à nutrição de camarões (Fernández Gimenez *et al.*, 2001), pois as propriedades das enzimas associadas a informações fisio-morfológicas do trato digestivo podem ajudar no entendimento e

determinação da capacidade digestiva destes organismos (Vega-Villasante *et al.*, 1995). Por causa da importância das enzimas digestivas na utilização dos nutrientes (Fernández *et al.*, 1997), o entendimento do modo como elas atuam é importante, servindo de base para uma correta formulação de rações com os componentes adequados para o cultivo de camarões marinhos (Muhlia-Almazán *et al.*, 2003), uma vez que a digestão é responsável direta pelo aproveitamento de monômeros moleculares utilizados nos processos metabólicos.

Dentre as enzimas digestivas de organismos aquáticos estudadas, as proteases são as que possuem maior destaque na literatura internacional, principalmente porque as proteínas são nutrientes indispensáveis para a estrutura funcional dos camarões. Uma vez que as proteínas estão sendo continuamente usadas para crescimento e reparo de tecidos, um contínuo suplemento de proteínas ou seus aminoácidos constituintes são necessários. Vale também destacar que as proteínas são os componentes principais e mais caros da alimentação de camarões. Portanto, estudos sobre a nutrição de camarões normalmente começam com a investigação de um nível adequado de proteínas na composição das rações. Como conseqüência, este é o principal nutriente pesquisado em estudos nutricionais com espécies de camarões peneídeos (Shiau, 1998).

As proteases digestivas mais importantes são tripsina, quimotripsina e aminopeptidases. A tripsina e a quimotripsina são endoproteases, ou seja, clivam as ligações peptídicas dentro da proteína, enquanto que aminopeptidases são exoproteases, isto é, clivam resíduos de aminoácidos na posição N-terminal da proteína (Gonzales & Robert-Baudouy, 1996).

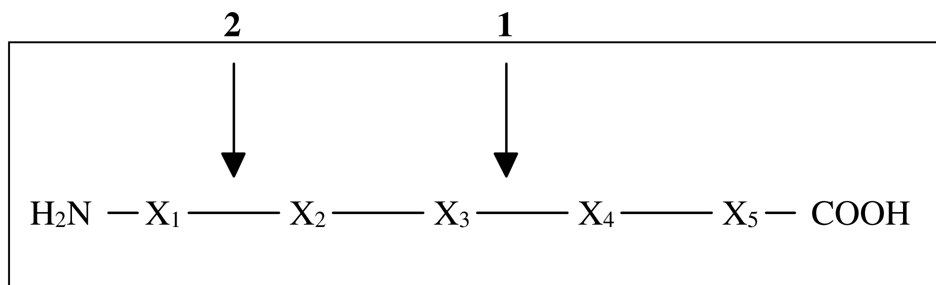

Figura 2: Classificação das proteases: Endoproteases clivam ligações peptídicas dentro da proteína (1). Exoproteases, mais especificamente as aminopeptidases, clivam resíduos localizados na posição N-terminal da proteína (2). Aminoácidos (X). Figura modificada de Gonzales & Robert-Baudouy (1996).

A tripsina geralmente é a protease mais abundante no sistema digestivo de crustáceos (Fernández Gimenez *et al.*, 2002). Alguns autores têm enfatizado a importância desta enzima em peneídeos e estimam que sua contribuição para a digestão protéica está em torno de 60% (Sánchez-Paz *et al.*, 2003). Ela é um membro da família das serino proteases, as quais são caracterizadas por um mecanismo catalítico comum, envolvendo a presença de uma tríade catalítica composta de resíduos específicos: serina, histidina e ácido aspártico (Klein *et al.*, 1996). Esta enzima cliva as ligações peptídicas no lado carboxila de resíduos de aminoácidos carregados positivamente como arginina e lisina (Figura 3) (Komklao *et al.*, 2007), sendo importantes em muitos processos biológicos como: digestão protéica propriamente dita, ativação de zimogênios e mediação entre a ingestão do alimento e a assimilação dos nutrientes (Sainz *et al.*, 2004). Vale também destacar a ampla aplicabilidade industrial de tripsinas (Klein *et al.*, 1996). Tais fatos têm feito destas enzimas as mais estudadas em organismos aquáticos.

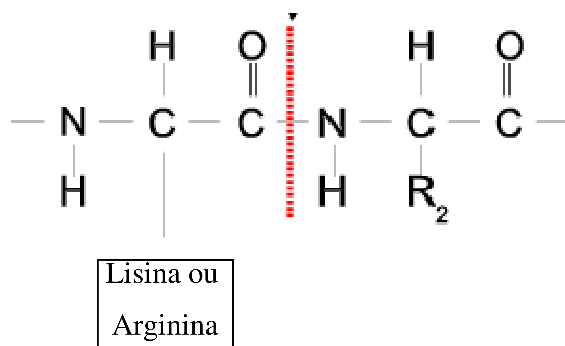

Figura 3: Sítio de hidrólise específico para tripsina.

Quimotripsina parece ser a segunda enzima mais abundante no sistema digestivo de crustáceos considerando a atividade proteolítica (García-Carreño *et al.*, 1994). Esta protease é uma endopeptidase a que catalisa a hidrólise de ligações peptídicas de proteínas na porção carboxila de aminoácidos aromáticos como: fenilalanina, tirosina e triptofano (Figura 4) e também substratos sintéticos, tais como SAPNA (De Vecchi & Coppes, 1996; Viparelli *et al.*, 2001; Abuin *et al.*, 2004; Castillo-Yañez *et al.*, 2006).

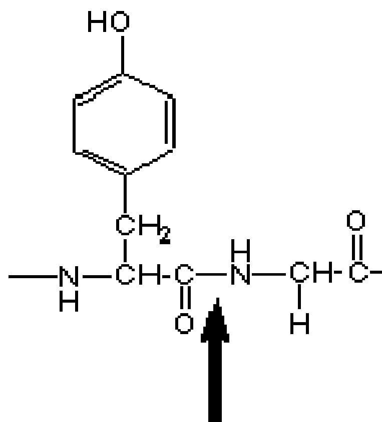

Figura 4: Sítio de hidrólise específico para quimotripsina.

As aminopeptidases são enzimas geralmente inespecíficas que catalisam a hidrólise em ligações peptídicas na posição N-terminal de proteínas liberando pequenos peptídeos e aminoácidos livres (Gonzales & Robert-Baudouy, 1996). Essas enzimas atuam também catalisando a hidrólise de substratos artificiais tais como aminoacil-β-naftilamida (AA-NA) e aminoacil-p-nitroanilida (AA-Nan). Elas estão amplamente distribuídas na natureza e apresentam importâncias biológicas e médicas por causa da sua função na degradação de proteínas (Oliveira *et al.*, 1999).

Apesar de todo o potencial de *Farfantepenaeus subtilis* e *Farfantepenaeus paulensis*, ainda existe uma lacuna no que diz respeito aos conhecimentos da biologia destas espécies, em particular com respeito às enzimas digestivas, que desempenham considerável relevância no sentido de otimização da relação dieta/atividade digestiva, contribuindo para a minimização do ônus econômico e ecológico com o mau gerenciamento dietário. Tais parâmetros já estão bem definidos para outras espécies (Lemos *et al.*, 1999; 2002; Muhlia-Almazán *et al.*, 2003). Portanto, o estudo de alguns aspectos da fisiologia digestiva de *F. subtilis* e *F. paulensis*, pode fornecer informações para pesquisas direcionadas à relação entre a capacidade digestiva e a alimentação de forma a viabilizar o cultivo destas espécies.

2. OBJETIVOS

2.1. Geral

Caracterizar endoproteases e exoproteases digestivas visando à contribuição para o conhecimento da digestão protéica das espécies nativas *Farfantepenaeus subtilis* e *Farfantepenaeus paulensis*.

2.2. Específicos

- Identificar proteases nas espécies citadas utilizando substratos específicos e inespecíficos e inibidores específicos;

- Caracterizar essas proteases utilizando SDS-PAGE e zimogramas e;

- Determinar parâmetros físico-químicos dessas enzimas tais como pH ótimo, temperatura ótima e estabilidade térmica.

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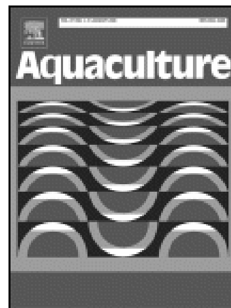
4. CAPÍTULO I –DIGESTIVE PROTEASES FROM THE HEPATOPANCREAS OF THE SOUTHERN BROWN SHRIMP *Farfantepenaeus subtilis*

ESTE ARTIGO SERÁ SUBMETIDO À RESVISTA INTERNACIONAL AQUACULTURE



Digestive proteases from the hepatopancreas of the Southern brown shrimp (*Farfantepenaeus subtilis*)

D. S. Buarque[a,b], F. M. S. Santos[a,b], I. P. G. Amaral[a,b], P. F. Castro[c], S. M. Oliveira[d], K. B. Alves[d], L. B. Carvalho Jr[a,b], R. S. Bezerra[a]*

[a] Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica, Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife-PE, Brazil

[b] Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Cidade Universitária, 50670-901, Recife-PE, Brazil

[c] Embrapa Meio-Norte, Caixa Postal 341, 64200-970, Parnaíba – PI, Brazil

[d] Departamento de Bioquímica, Universidade Federal de São Paulo, Vila Clementino, 04044-020, São Paulo-SP, Brazil

Ranilson S. Bezerra.

Laboratório de Enzimologia – LABENZ, Departamento de Bioquímica, Universidade Federal de Pernambuco. Cidade Universitária, 50670-420, Recife-PE, Brazil,

Tel.: + 55-81-21268540, Fax: + 55-81-21268576

E-mail address: ransoube@uol.com.br

Abstract

Studies concerning the characterization of digestive proteases are important for understanding the digestive physiology of any potential aquaculture species. They enable the correct management of dietary necessities and the optimization of the use of nutrients, thus reducing levels of not assimilated feed and metabolic excretions. Hence, this study sets out to examine proteases from the hepatopancreas in juvenile and adult brown shrimps (*Farfantepenaeus subtilis*). No significant differences were recorded in proteolytic activities between the life stages using azocasein, Leu-p-Nan and aminoacyl of β-naphthylamide as substrates ($p \geq 0.05$). Trypsin-like (benzoyl-DL-arginine p-nitroanilide - BAPNA) and chymotrypsin-like (Succinyl alanine alanine proline phenylalanine p-nitroanilide - SAPNA) activities were higher in extracts of juvenile than in adult shrimps ($p < 0.05$). The highest activities among aminoacyl of β-naphthylamide substrates was found using alanine, arginine, leucine and lysine-β-naphthylamide. The proteolytic activity of juvenile and adult shrimps was strongly inhibited by tosyl-lysine chloromethyl ketone (TLCK) and benzamidine, specific trypsin inhibitors. The optimum temperature for trypsin-like and chymotrypsin-like activities in both life stages was the same (55ºC), while adults (45ºC) and juveniles (55ºC) showed a significant difference in optimum temperature for leucine aminopeptidase activity. However, trypsin retained about 15% of the initial activity while chymotrypsin-like and leucine aminopeptidase-like retained 60% and 45% of activity, respectively. The thermal stability zymogram showed a similar proteolytic band pattern between juvenile and adult shrimps, except that juvenile shrimps presented a thermostable proteolytic band at 65ºC. All bands were inhibited by phenyl-methyl-sulfonyl-fluoride (PMSF) in both life stages. The use of TLCK and benzamidine showed a strong inhibition of the proteolytic bands. Slight qualitative and quantitative differences between the proteases pattern of *F. subtilis* juvenile and adult were observed.

Keywords: Aminopeptidases; Chymotrypsin; *Farfantepenaeus subtilis*; Southern brown shrimp; Trypsin.

1. Introduction

The Southern brown shrimp *Farfantepenaeus subtilis* is native to the Atlantic coast of Central and South America, from Cuba down to Rio de Janeiro, and was one of the first species to be farmed in Brazil, along with *F. brasiliensis*, *F. paulensis* and *Litopenaeus schmitti*. The Southern brown shrimp exhibits benthic omnivorous opportunistic feeding habits under semi-intensive conditions, although polychaetes and calanoid copepods seem to be favored during all growth stages (Nunes and Parsons, 2000).

Despite its farming potential and attractive market features, the culture of *F. subtilis* in Brazilian semi-intensive conditions has failed, mainly due to the low yields obtained. Studies carried out by Brazilian farmers reported a food conversion ratio (FCR) ranging from 2.88 to 3.44 and an untypical growth performance, thus generating low productivities. Shrimps presented a slowdown in their growth rate after reaching 6g of body weight. This would seem to suggest that poor results may be related to nutritional problems and ontogenetic changes in digestive enzyme metabolism (Maia and Nunes, 2003).

In fact, comprehension of the digestive physiology and nutrient digestibility remains a problem for the *F. subtilis* culture. Knowledge concerning the digestive system of this species can provide information applicable to food utilization. Thus, the identification and characterization of digestive enzymes during shrimp growth is an important step towards understanding the digestive mechanisms (Lemos et al., 2000; López-López et al., 2005) and formulating diets that promote better growth responses, since feed can be designed according

to shrimp digestion capacities (Férnandez Gimenez et al., 2001; Gamboa-Delgado et al., 2003).

Several studies have indicated properties of digestive enzymes in shrimp and other crustacean: proteases (Oh et al., 2000; Córdova-Murueta et al., 2003; Muhlia-Almazan et al., 2003; Córdova-Murueta et al., 2004; Sainz et al., 2004), carbohydrases (Ribeiro and Jones, 2000; Brito et al., 2001; Guzman et al., 2001; Aguilar-Quaresma and Sugai, 2005; Gaxiola et al., 2005), lipases (Moss et al., 2001; Gamboa-Delgado et al., 2003; López-López et al., 2003; 2005) and digestibility of feed ingredients (Ezquerra et al., 1997; 1998; Lemos et al., 2000; 2004). However, synthesis regulation and enzymatic activity are species-specific (Fernández Gimenez et al., 2002) and it is therefore not possible to extrapolate characteristics between species. Thus, there is an increasing need to study a native shrimp species with high market features, such as the *F. subtilis* (Nunes et al., 1996). This work describes the investigation and properties of some digestive proteases from the hepatopancreas of the brown shrimp *F. subtilis* during different life stages.

2. Materials and methods

2.1. Materials

F. subtilis specimens were obtained from commercial fishery along the coast of Barra de Serinhanhém (8º 36’ S; 35º 1’ W), 100km from Recife, in the state of Pernambuco, Brazil. All reagents used in enzymatic assays were from an analytical grade purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

2.2. Enzyme extraction and total soluble protein determination

The shrimps were transported alive to the Laboratório de Enzimologia (LABENZ) at the Universidade Federal de Pernambuco. Shrimps were classified into juveniles and adults

according to descriptions of Nunes and Parsons (2000). The hepatopancreas of 20 juveniles (5.93 ± 0.69 g wet weight) and 20 adults (13.26 ± 0.60 g wet weight) were readily dissected from the decapted shrimps. For each life stage, four groups of five hepatopancreas were homogenized (40 mg/mL) in chilled 0.15 M NaCl, using a tissue homogenizer (Bodine Electric Company – Chicago, USA). Homogenates were centrifuged at 10,000 xg for 25 min at 4°C to remove lipid and tissue debris and supernatants (crude enzyme extract) were stored at –20°C for further utilization. The total soluble protein was determined as described by Bradford (1976), using bovine serum albumin as standard protein.

2.3. Unspecific enzyme assays

Unspecific proteolytic activity was assayed using azocasein as substrate in a microcentrifuge tube (Bezerra et al., 2005). Triplicate samples of each enzyme extract (30 μL) were incubated with 1% azocasein (50 μL) dissolved in 0.1 M Tris-HCl pH 8.0, for 60 min at 25°C. Following, 10% trichloroacetic acid (120 μL) was added to stop the reaction and the mixture was centrifuged at 8000 xg, for 5 min. The supernatant (70 μL) was mixed with 1 M NaOH (130 μL) and absorbance was measured in a microtiter plate reader (Bio-Rad 680) at 450 nm against a similarly prepared blank, except that 0.9% (w/v) NaCl replaced the crude extract sample. Previous experiments showed that for the first 60 min the reaction carried out under the above mentioned conditions follows first order kinetics. Protease activities were expressed as units per mg of protein. One unit (U) of enzymatic activity was defined as the amount of enzyme required to hydrolyse azocasein and to produce a 0.001 change in absorbance per mL per minute.

2.4. Specific enzyme assays

Trypsin, chymotrypsin and leucine aminopeptidase activities were determined in a 96 well microtiter plate using benzoyl-DL-arginine p-nitroanilide (BAPNA), Succinyl alanine

alanine proline phenylalanine p-nitroanilide (SAPNA) and leucine-p-nitroanilide (Leu-p-Nan) as specific substrates, respectively (Bezerra et al., 2005). Triplicate samples of enzyme extracts (30 µL) were incubated with either 4mM BAPNA, SAPNA or Leu-p-Nan (30 µL), dissolved in dimethylsulphoxide (DMSO), and 0.1 M Tris-HCL (140 µL) pH 8.0. The reactions occurred at 25ºC for 15 min and were recorded at 405 nm using a microplate reader (BIO-RAD 680). The activity was expressed as protease milliunits per milligram of protein. One unit (U) of activity was defined as the amount of enzyme required to produce one µmol of p-nitroaniline per minute.

Aminopeptidase activity was also evaluated using aminoacyl β-naphthylamide (AA-NA) as substrates. The substrates used were: AA-NA of Ala, Arg, Leu, Phe, Val, Ser, Gli, Ile, Tyr, His, Lys and Glu. The procedure adapted from Oliveira et al. (1999) was carried out by incubating 4.2 mM substrate (50 µL), 50 mM sodium phosphate buffer pH 7.0 (600 µL) and H_2O (50 µL) at 37ºC. After temperature equilibration the enzyme (50 µL) was added. After 120 minutes, the reaction was stopped by adding fresh Garnet reagent (250 µL) prepared in 0.2 M sodium acetate buffer, pH 4.2, containing 10% v/v Tween 20. Absorbance was measured at 525 nm and the amount of β-naphthylamine was determined using a standard curve of β-naphthylamine. The activity was expressed as protease milliunits per milligram of protein. One unit of enzymatic activity was defined as the amount of enzyme required to hydrolyze one µmol of β-naphthylamine per minute.

2.5. Inhibition assays

The following inhibitors prepared in DMSO at 1mM final concentration were used: Phenyl-methyl-sulfonyl-fluoride (PMSF – serine proteases inhibitor), tosyl-lysine chloromethyl ketone (TLCK) and benzamidine (both trypsin inhibitors), Tosyl-phenylalanine chloromethyl ketone (TPCK – chymotrypsin inhibitor) and bestatin (aminopeptidase

inhibitor) (Bezerra et al., 2005). Triplicate samples of enzyme extract (25 μL) and inhibitors (25 μL) were placed in a well of a microtiter plate and incubated at 25ºC for 15 min. Volumes were then adjusted to 170 μL with 0.1 M Tris-HCl pH 8.0 and the respective substrate (BAPNA – PMSF, TLCK and benzamidine; SAPNA – PMSF and TPCK; Leu-p-Nan - bestatin) and the proteolytic activity were determined as described above. The enzyme and substrate blank were similarly assayed without enzyme and substrate solution, respectively. The 100% values were established using DMSO without inhibitors.

2.6. Physical chemical properties

The effects of pH and temperature on proteolytic activity of the *F. subtilis* enzyme extract were evaluated as described above, using 0.1 M Tris-HCl buffer pH ranging from 7.2 to 9.0 and temperatures ranging from 25 to 85ºC, respectively. Thermal stability was evaluated by assaying enzyme activities at 25ºC after pre-incubation for 30 min at temperatures ranging from 25 to 85ºC (Bezerra et al., 2005).

2.7. Electrophoresis SDS-PAGE and zymograms

Proteases from *F. subtilis* were studied by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% (w/v) stacking gel and a 12.5% (w/v) separating gel (Laemmli, 1970). Enzyme preparations and molecular weight markers (15μL) (Ovoalbumin - 46 kDa, glyceraldehyde 3-phosphate dehydrogenase - 36 kDa, carbonic anhidrase - 29 kDa, trypsinogen - 24 kDa and α-lactalbumin - 14.2 kDa) were applied in each lane of a vertical electrophoresis device (BIO-RAD). The gels were stained for protein overnight in 0.18% (w/v) Coomassie Brilliant Blue R250 prepared in acid acetic; methanol (10:25% v/v) and the background of the gel was destained in acetic acid; methanol (10:25% v/v). Electrophoresis was performed at a constant current of 15 mA per gel, at 4°C.

Zymograms were also carried out, in accordance with Garcia-Carreño et al. (1993). After electrophoresis, the gels were immersed in 2.5% (100 mL) Triton X-100 in 0.1 M Tris-HCl pH 8.0 for 30 min at 4°C to remove the SDS. The Triton X-100 was removed by washing the gels three times with 100 mL of 0.1 M Tris-HCl buffer, pH 8.0. Then, the SDS and Triton X-100 free gels were incubated with 100 mL 3% casein (w/v) in 0.1 M Tris-HCl pH 8.0, for 30 min at 4°C. The temperature was raised to 25°C and maintained for 90 min to allow the digestion of casein by the active fractions. Finally, the gels were stained and destained as described above. The thermal stability was also determined using the caseinolytic zymogram. It was used the same methodology described above, except that samples were pre-incubated at temperatures ranging from 25°C to 75°C. For zymogram of enzymatic inhibition, samples were pre-incubated with serine protease and trypsin inhibitors as described above (inhibition assays). Samples incubated with proteinase inhibitors were compared with control (without inhibitors). Decrease of the intensity or evanishment of the bands indicated inhibiton of proteolytic activity, identifying the type of enzyme.

2.8. Statistics

Data (mean±standard deviation) processing was carried out using Microcal Origin 6.0. Differences among means were analyzed by Student's test. Differences were considered significant when $p<0.05$ (Zar, 1984).

3. Results

The proteolytic activities of juvenile and adult *F. subtilis* are presented in Table 1. Total proteolytic activity did not differ ($p\geq0.05$) between juveniles and adults. The use of specific substrates evidenced the presence of trypsin-like, chymotrypsin-like and leucine aminopeptidase-like enzymes. Trypsin-like (BAPNA) and chimotrypsin-like (SAPNA) activity was significantly higher ($p<0.05$) in juveniles than in adults. Chymotrypsin-like

activity was almost twice higher in juveniles than in adults. The presence of aminopeptidases was more evident with β-naphthylamide substrates (Table 1). It was possible to observe activity for all employed aminoacyl of β-naphthylamide substrates. Higher activities were found for basic (Arg-, Lys-) and nonpolar (Ala-, Leu-) substrates. On the other hand, lower activities were observed for aromatic (Tyr-), polar uncharged (Ser-), and nonpolar (Gly-) substrates. It is noteworthy that some of the aminoacyl β-naphthylamide substrates employed herein revealed negligible activity, namely Glu-, Ile-, Phe-, His-, and Val-β-naphthylamide. There were no statistical differences in the proteolytic activities ($p \geq 0.05$) between juveniles and adults using aminoacyl β-naphthylamide as substrates. It was also detected a positive correlation between recommended level of essential amino acids in penaeid shrimp feed and the aminopeptidasic activity using aminoacyl β-naphthylamide as substrates (Fig. 1).

Digestive proteases were partially inhibited by PMSF (using BAPNA and SAPNA as substrates) in both life stages (Table 2). TLCK and benzamidine presented a strong effect of trypsin activity inhibition on juveniles (91.70±0.58% and 89.81±0.21%, respectively) and adults (92.20±0.21 and 89.91±0.15%, respectively). TPCK affected chymotrypsin-like activity in both life stages. Leucine aminopeptidase activity was inhibited by bestatin at a rate of 81.49%±0.02 for juveniles and 84.21%±0.01 for adults.

The highest trypsin-like activities were obtained at a pH range from 8.0 to 9.0, with an optimum level at pH 8.5 for both juveniles and adults. Maximum chymotrypsin-like activity was observed at pH 8.5 for juveniles and 8.0 for adults. Leucine aminopeptidase presented optimum pH 8.0 in both life stages (Fig. 2).

The influence of temperature on proteolytic activity is shown in Fig. 3. Trypsin-like enzymes presented an optimum temperature at 55°C for both juvenile and adult *F. subtilis* under experimental conditions. Trypsin-like activity dropped sharply when submitted to

temperatures higher than 55°C. This effect was more noticeable in adult shrimps. The same result was observed for chymotrypsin-like enzymes. The optimum temperature of *F. subtilis* leucine aminopeptidase was 45°C and 55°C for juveniles and adults, respectively.

It was observed that residual trypsin-like activity was reduced to 15% after a 30 min heat treatment at 55°C (optimum temperature) in both juveniles and adults (Fig. 4A). A similar profile was obtained for the thermal stability of leucine aminopeptidase-like activity in both juvenile and adult specimens (Fig 4C). Chymotrypsin-like enzymes showed higher heat resistance than trypsin-like and leucine aminopeptidase-like enzymes (Fig 4B). No significant loss of activity occurred up to 35°C. At 60°C, the activiy decreased towards 60% of initial value.

Proteins from the hepatopancreas of juvenile and adult *F. subtilis* were analyzed by electrophoresis (Fig. 5). A common pattern was observed concerning the number of bands in both life stages. Six bands ranging from 15.3 to 42.2 kDa were detected. Three bands were found to have molecular weights higher than 45 kDa. On the other hand, one band presented a molecular weight lower than 14.2 kDa.

Juvenile and adult proteolytic activities were detected in substrate gel electrophoresis (Fig. 6). Adults presented six intense activity bands at 25°C while an extra band was detected in juveniles (white dashed arrow, Fig. 6). This proteolytic band was also evident in Fig. 7A (black arrow). From 35°C to 55°C the number and intensity pattern of bands were similar for both life stages. One band remained active in juveniles even at temperatures as high as 65°C (white arrow, Fig. 6).

Enzyme inhibition zymogram is shown in Fig. 7. All proteolytic bands were inhibited by PMSF (lane 1) in juvenile and adult extracts, indicating that most bands should be serine proteases. No reaction was recorded in just two bands with TLCK (lane 2) and in three bands

with benzamidine (lane 3), suggesting a strong presence of trypsin-like in the *F. subtilis* enzymatic extract. By comparing these results to those recorded in Fig. 6, it is possible to note that the juvenile thermostable proteolytic band (white arrow, Fig. 7A) was inhibited by PMSF, TLCK and benzamidine. The proteolytic bands that were not inhibited by trypsin inhibitors presented a stronger intensity (white dashed arrows, Fig 7A).

4. Discussion

Trypsin-like and chymotrypsin-like enzymes were identified in both *F. subtilis* juvenile and adult life stages. The higher trypsin- and chymotrypsin-like activities in juvenile shrimps could be related to the faster metabolism of younger organisms. Generally, the crustacean digestive system presents a high concentration of serine proteases, mainly trypsin and chymotrypsin (Fernández et al., 1997). Trypsin also plays an important role in digestion through the activation of zymogens of both itself and other endopeptidases (Natalia et al., 2004).

In this work, the presence of aminopeptidases was also observed in both life stages. Little information is available about aminopeptidases in shrimps. Thus, in order to provide a greater understanding of these enzymes, leucine p-nitroanilide and aminoacyl β-naphthylamide substrates were used. Aminopeptidases of hepatopancreas of *F. subtilis* juveniles and adults were capable to strongly hydrolyse alanine, arginine, leucine, lysine and serine β-naphthylamide substrates. A higher hydrolysis of substrates containing amino acids required at higher levels in shrimp diets was observed, namely arginine (5.8% of crude protein-CP), leucine (5.4% CP) and lysine (5.3% CP) (Guillaume, 1997). These amino acids correlated with the aminopeptidasic activity using some β-naphthylamide substrates (Arg-, Leu-, Lys-, Phe- and Val-). The results corroborate the requirements reported in literature, since lysine and arginine are described as the most limiting essential amino acids in commercial shrimp

feeds (Fox et al., 1995). The correlation between recommended levels of some dietary components such as lysine and arginine and high aminopeptidasic activity can be related to efficient digestion and incorporation of these key nutrients (Lemos and Nunes, in press). Heu et al. (2003) also found high aminopeptidase activities for the same amino acids in *Pandalus borealis* and *Trachypena curvirostris* processing residue.

The most relevant proteases from decapod crustacean belong to the serine class and are inhibited by PMSF (García-Carreño et al., 1994; Lemos et al., 1999; 2002). The results show that serine proteases are present in hepatopancreas tissues of *F. subtilis*. Moreover, the strong inhibition of TLCK and benzamidine on the proteolytic activity in both studied life stages indicates classical tryptic activity (traditional mammalian trypsin). Chymotryptic activity was partially inhibited by TPCK on crude extract of both life stages. Similar results were obtained on extract of *Artemesia longinaris* in premolt and intermolt, respectively (Fernandéz Gimenez et al., 2002). The high inhibition by bestatin also evidenced the presence of aminopeptidases.

Crustacean proteases generally show the highest activities in the range between pH 5.5 and 9.0 (García-Carreño, 1992) and in trypsin from pH 7.0 to 9.0 (Maeda-Martínez et al., 2000). The optimum pH for trypsin-like enzymes in *F. subtilis* juveniles and adults in this study falls within this interval. Maeda-Martínez et al. (2000) reported that trypsin of crustacean *Triops sp.* presented maximum activity at pH 8.5 using BAPNA as substrate. Jiang et al. (1991) encountered three enzymes from *Penaeus monodon* that presented the highest activity at pH 8.0 using p-toluenesulfonyl-$_{\text{L}}$-arginine methyl ester (TAME) as substrate.

Trypsin-like enzymes from the hepatopancreas of both juvenile and adult *F. subtilis* showed the highest proteolytic activity at similar temperatures, namely 55°C at pH 8.0. These values correspond to those recorded in other crustaceans (from 50°C to 60°C) (Maeda-Martínez et al., 2000; Sainz et al., 2004). Jiang et al. (1991) reported that two trypsin enzymes

from *Penaeus monodon* at pH 8.0 showed a maximum activity at 65°C while a third trypsin exhibited the highest activity at 55°C with the same pH reading, using casein as substrate. However, trypsin-like enzymes from juvenile and adult *F. subtilis* retained about 15% of their activity after incubation for 30 min at 55°C (Fig. 4). Although trypsin-like presented the maximum activity at 55°C in both life stages, its thermal stability was low at the same temperature, suggesting that most of this enzyme should have been denaturated. Saborowski et al. (2004) demonstrated that the trypsin activity from the marine crab *Cancer pagurus* decreased to 30% of initial activity after 60 min at 50°C. Bezerra et al. (2001; 2005) and Souza et al. (2007) demonstrated that trypsins from tropical fish are more thermostable than *F. subtilis* proteases.

Chymotrypsin-like enzymes also presented maximum proteolytic activity in the alkaline range. Chymotrypsin from the gut of *Daphnia magna* also had optimum pH in the alkaline range (pH 8 to 10) for hydrolysis of SAPNA (Elert et al., 2004). Saborowski et al. (2004) demonstrated that chymotrypsin from gastric fluid of crab *Cancer pagurus* showed maximum activity in a range around pH 7.0 using the same substrate.

There are few information concerning heat treatment and temperature resistance of chymotrypsins from crustaceans. Chymotrypsin activity from gastric fluid of crab *Cancer pagurus* was extinguished after incubation at 60°C for 20 min (Saborowski et al., 2004). Therefore, chymotrypsin-like of *C. pagurus* seems to be less thermostable than the same enzyme in *F. subtilis* (about 40% of initial value after a 30 mim heat treatment at 60°C).

Many authors have studied aminopeptidases from fish (Sabapathy and Teo, 1993; Tengjaroenkul et al., 2000; 2002; Natalia et al., 2004; Ma et al., 2005; Refstie et al., 2006). This demonstrates the importance of understanding the role of aminopeptidases in the protein digestion of aquatic organisms. However, there is a lack of information concerning

aminopeptidases from shrimps in relation to their physical-chemical characterization. Further studies are required in order to compare the physical chemical effects on aminopeptidases within several shrimp species. Regarding the properties of leucine aminopeptidase from the hepatopancreas of juvenile and adult *F. subtilis*, pH and optima temperature were found to be 8.0 and 50-55^{0}C, respectively, and indicated a similar temperature denaturation profile being completely inactivated at 80^{0}C.

In the electrophoresis (SDS-PAGE) of the extracts from the hepatopancreas of juvenile and adult *F. subtilis* a similar pattern was observed in both samples (Fig. 5). Two bands are well visualized between the molecular weight range of 24-29 kDa which are equivalent to the trypsin-like enzyme molecular weight reported in the literature for other aquatic animals (Kolodziejska and Sikorski, 1996).

The thermal stability of proteolytic enzymes from *F. subtilis* is also shown in zymograms and the results demonstrated similar band profile in both studied life stages. However, one slight band with proteolytic activity at 65°C was observed in juveniles (white arrow, Fig. 6), suggesting that this enzyme remains active even under adverse temperature conditions. Moreover, one extra band was also observed in the crude extract from juvenile specimens indicating the presence of one more protease in the hepatopancreas of *F. subtilis* (white dashed arrow, Fig. 6). It is possible to note that this band was inhibited by PMSF, TLCK and benzamidine (white arrow, Fig. 7), suggesting that it is a trypsin-like enzyme. All bands remained active until 55°C in both life stages, even though the quantitative determination of proteolytic activity was low at the same temperature. In fact, the zymogram technique is more sensitive than the quantitative assays using soluble substrates (Lemos et al., 2000).

Proteolytic bands inhibited by both PMSF (serine protease inhibitor) and TLCK or benzamidine (trypsin inhibitors) correspond to trypsin, which is a key enzyme in proteolytic

digestion. Inhibition only by PMSF indicate the presence of chymotrypsin (Lemos et al., 2002), another proteolytic enzyme present in the hepatopancreas of penaeid shrimps (Muhlia-Almazán and García-Carreño, 2002).

Our results indicated the presence of several trypsin forms and suggested the presence of at least two chymotrypsin forms in both life stages. Curiously, adults of closed thelycum species such as *F. paulensis* display a higher number of trypsin than chymotrypsin forms. This shrimp presents 4 trypsin and 3 chymotrypsin forms (Lemos et al., 1999). It was also noted in our results that chymotrypsin bands were more intense when the extract was treated with trypsin inhibitors. This may occur as an alternative to the digestion of a food with a high rate of trypsin inhibitors.

This study showed a large diversity of proteases in the hepatopancreas of *F. subtilis*, with the presence of trypsin, chymotrypsin and aminopeptidases. The remarkable difference between life stages was the major chymotrypsin-like activity observed in crude extract of juveniles. These proteolytic enzymes revealed an optimum pH within the expected range for a decapod crustacean as described in the available literature. While the trypsins and leucine aminopetidases found in this study presented an optimum temperature of about 55ºC, they were not thermostable at this temperature. The presence of a high content of endo and exoproteases render protein digestion more efficient. It is also important to draw attention to the fact that a greater understanding of the digestive physiology of this species is essential in order to reduce environmental pollution. A badly managed dietary plan may lead to excessive feed loss and metabolic excretion, which is responsible for economical and ecological onus.

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Tables

Table 1: Proteolytic activity from hepatopancreas of juvenile and adult *F. subtilis* using specific and unspecific substrates.

Substrate	Life stage	
	Juvenile	Adult
Unspecific substrate (mU/mg)		
Azocasein	12.201^{a}±0.625	11.967^{a}±0.743
p-nitroanilide substrates (mU/mg)		
BAPNA	6.820^{a}± 0.140	5.130^{b}± 0.080
SAPNA	8.390^{a}±0.568	4.405^{b}±0.193
Leu-p-Nan	0.330^{a}±0.020	0.280^{a}±0.020
β-naphthylamide substrates (U/mg)		
Alanine	0.100^{a}±0.012	0.100^{a}±0.005
Arginine	0.100^{a}±0.010	0.100^{a}±0.008
Lysine	0.070^{a}±0.010	0.060^{a}±0.004
Leucine	0.060^{a}±0.003	0.050^{a}±0.010
Tyrosine	0.020^{a}±0.006	0.017^{a}±0.006
Serine	0.020^{a}±0.001	0.016^{a}±0.007
Glycine	0.010^{a}±0.001	0.008^{a}±0.004
Glutamic Acid	0.007^{a}±0.007	0.010^{a}±0.007
Isoleucine	0.007^{a}±0.003	0.009^{a}±0.005
Phenylalanine	0.005^{a}±0.005	0.005^{a}±0.001
Valine	0.005^{a}±0.006	0.004^{a}±0.006
Histidine	0.002^{a}±0.003	0.003^{a}±0.006

Values are shown in mean ± Standard Deviation (SD) of triplicate of four crude extracts obtained from five hepatopancreas each. Different italic letters show statistical differences ($p<0.05$). BAPNA: benzoyl-DL-arginine p-nitroanilide – Trypsin specific substrate; SAPNA: Succinyl alanine alanine proline phenylalanine p-nitroanilide; Leu-p-Nan: leucine p-nitroanilide – leucine aminopeptidase specific substrate.

Table 2: Effect of specific Inhibitors from hepatopancreas of juveniles and adults *Farfantepenaeus subtilis*.

Inhibitors	Enzymatic Inhibition (%)	
	Juvenile	Adults
PMSF[1]	52.35^{a}±0.338	56.72^{b}±0.363
PMSF[2]	55.23^{a}±0.724	46.19^{b}±0.178
TLCK	91.70^{a}±0.580	92.20^{b}±0.205
Benzamidine	88.54^{a}±0.213	$89,81^{b}$±0,147
TPCK	42.40^{a}±0.475	22.45^{b}±0.294
Bestatin	81.49^{a}±0.020	84.21^{a}±0.010

Values are shown in mean ± SD of triplicate of four crude extracts obtained from five hepatopancreas each. Different italic letters show statistical differences ($p<0.05$). The maximum specific proteolytic activity (100%) was 7.03 mU/mg for juveniles and 6.09 mU/mg for adults using BAPNA as substrate. 100% was 4.40 mU/mg for juveniles and 4.94 mU/mg for adults using SAPNA as substrate. 100% was 0.29 mU/mg for juveniles and 0.23 mU/mg for adults of *F. subtilis* using Leu-p-Nan as substrate.

PMSF: phenylmethylsulphonyl fluoride

TLCK: tosyl lysine chloromethyl Ketone

TPCK: Tosyl Phenylalanine Chloromethylketone

[1] PMSF Inhibition using BAPNA as substrate.

[2] PMSF inhibition using SAPNA as substrate.

Figures

Buarque, D. S. et al.

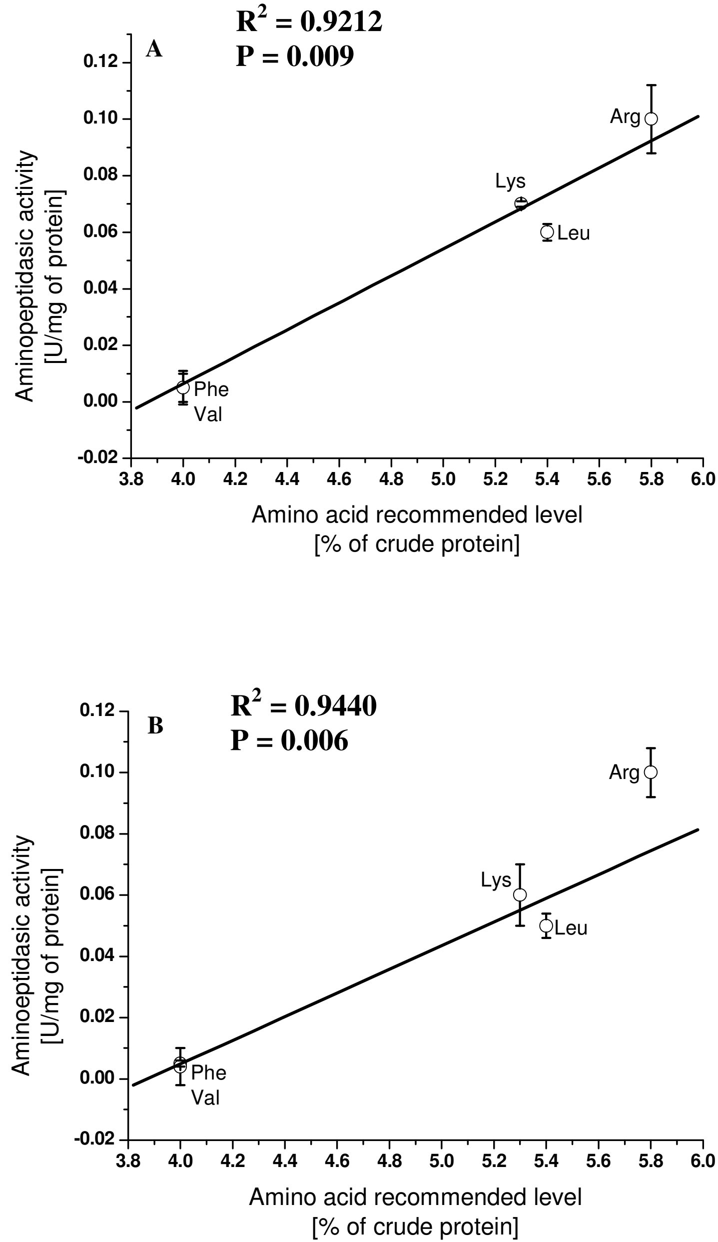


Figure 1

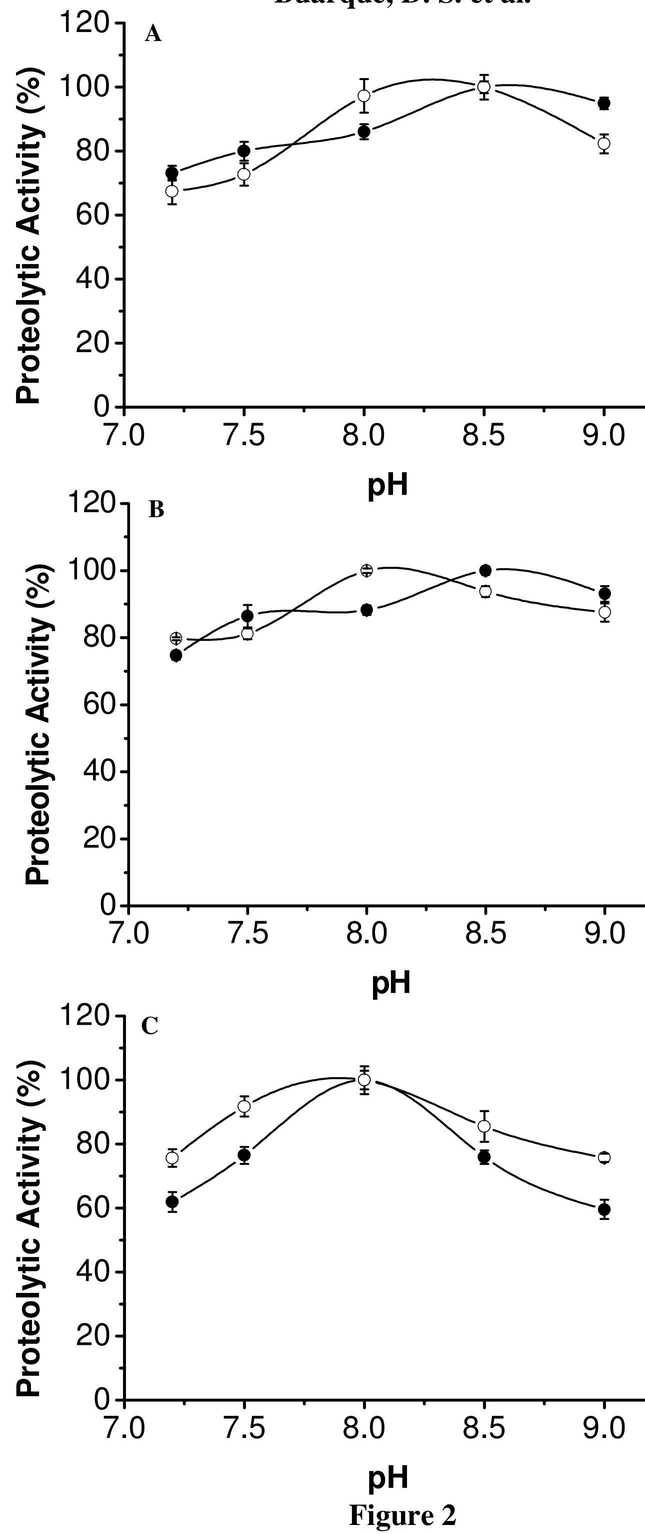


Figure 2

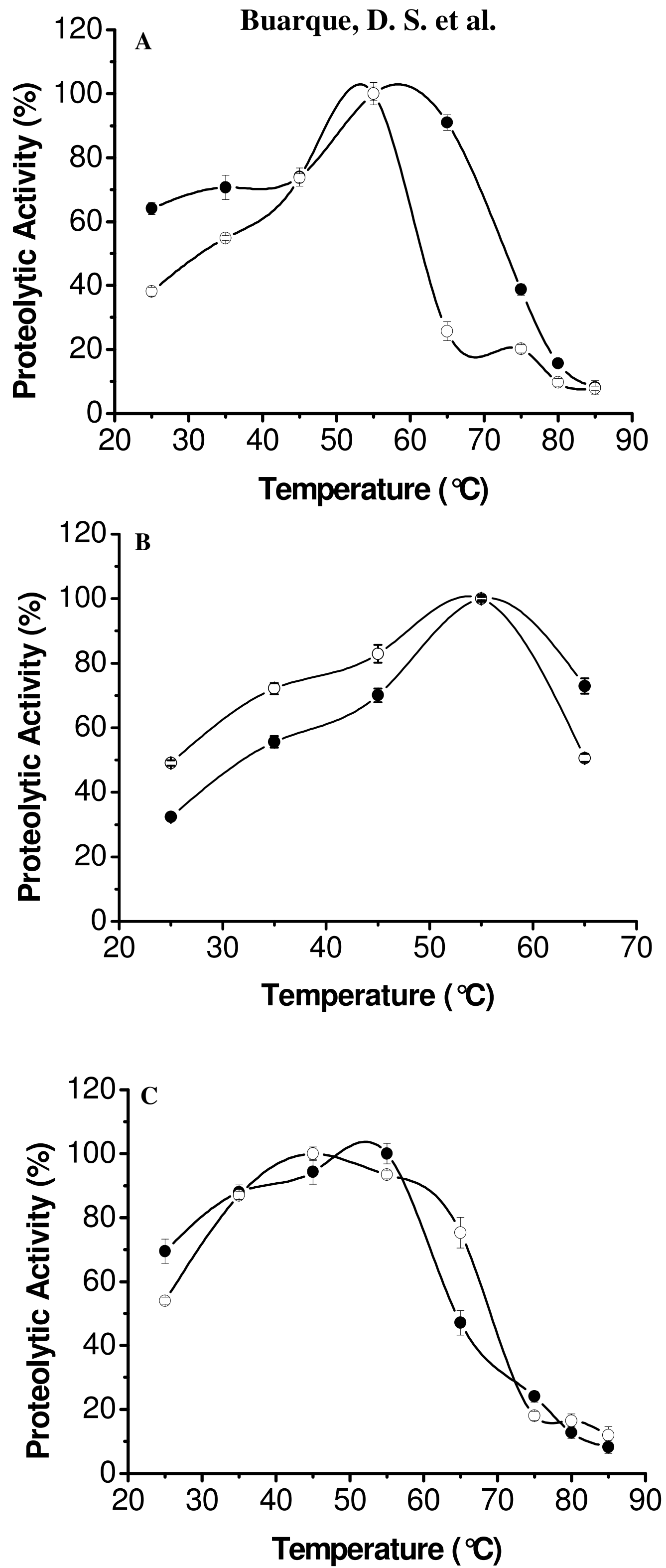


Figure 3

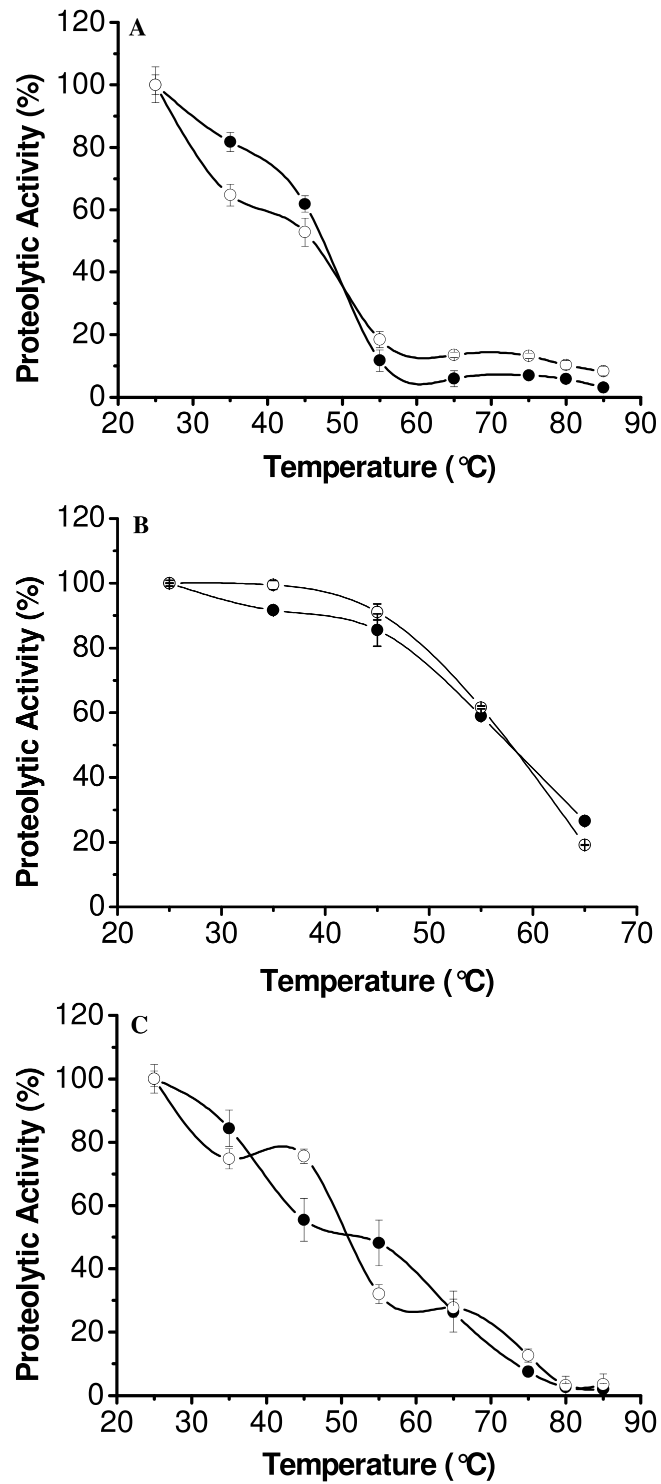
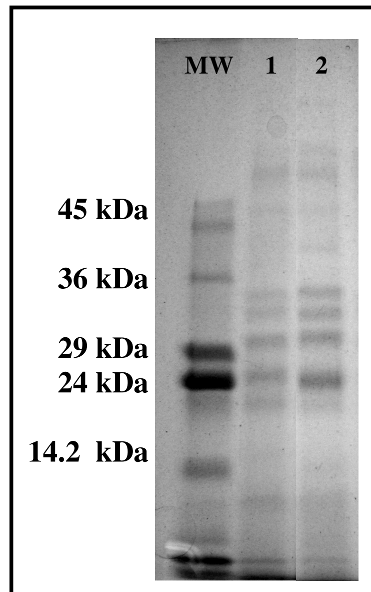


Figure 4



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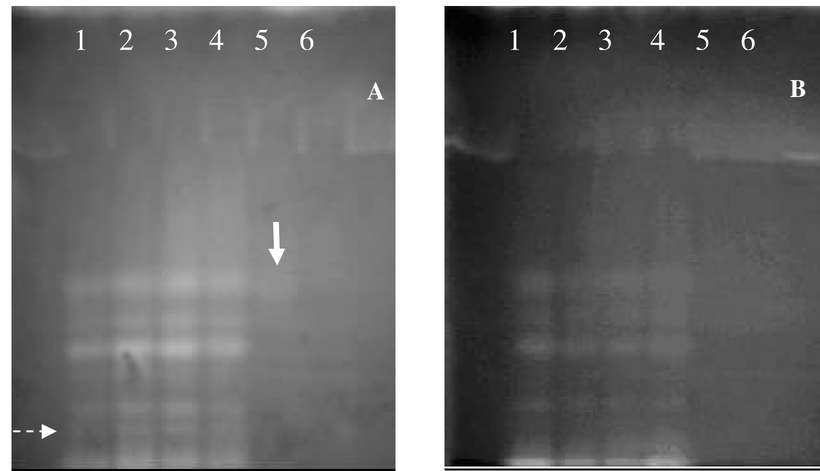

Figure 6

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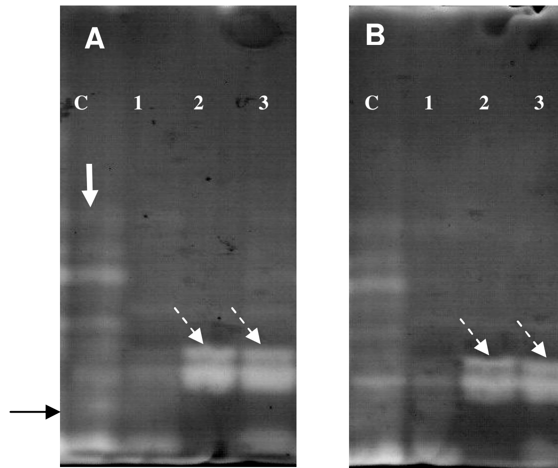

Figure 7

Figure captions

Fig 1: Correlation between aminopeptidasic activity of juveniles (**A**) and adults (**B**) of *F. subtilis* using aminoacyl β-naphthylamide as substrates and recommended level of essential amino acids for Penaeid shrimp feed. Values are shown in mean ± SD of triplicate of four crude extracts obtained from five hepatopancreas each. Level of amino acids expressed in % of crude protein: Arginine (5.8), Leucine (5.4), Lysine (5.3), Phenylalanine (4.0), Valine (4.0). *According to Guillaume (1997).

Fig. 2: Effects of pH on proteolytic activity of juveniles (●) and adults (o) of *F. subtilis* using BAPNA (**A**), SAPNA (**B**) and Leu-p-Nan (**C**) as substrates. Values are shown in mean ± SD of triplicate of four crude extracts obtained from five hepatopancreas each. The crude extract was incubated with the above substrates in different assays at the indicated pH for 15 min and reactions were measured at 405 nm. Values were expressed as percentages of the highest (100%) and were 7.20 mU/mg for juveniles and 5.15 mU/mg for adults using BApNA as substrate, 8.51 mU/mg for juveniles and 4.96 mU/mg for adults using SAPNA and 0.29 mU/mg for juveniles and 0.25 mU/mg for adults Leu-p-Nan as substrate respectively.

Fig. 3: Effects of temperature on proteolytic activity from juveniles (●) and adults (o) of *F. subtilis* using BAPNA (**A**), SAPNA (**B**) and Leu-p-Nan (**B**) as substrates. Values are shown in mean ± SD of triplicate of four crude extracts obtained from five hepatopancreas each. The crude extract was incubated with the above substrates in different assays at the indicated temperatures for 15 min and reactions were measured at 405 nm. Values were expressed as percentages of the highest (100%) and were 7.41 mU/mg for juveniles and 5.49 mU/mg for adults using BApNA as substrate, 46.09 mU/mg for juveniles and 28.02 mU/mg for adults

using SAPNA and 0.46 mU/mg for juveniles and 0.42 mU/mg for adults Leu-p-Nan as substrate respectively.

Fig. 4: Effects of thermal stability on proteolytic activity from juveniles (●) and adults (o) of *F. subtilis* using BAPNA (**A**), SAPNA (**B**) and Leu-p-Nan (**B**) as substrates. Values are shown in mean ± SD of triplicate of four crude extracts obtained from five hepatopancreas each. Thermal stability was determined by assaying its activity (25°C) after pre-incubation for 30 min at the indicated temperatures. Values were expressed as percentages of the highest (100%) and were 7.14 mU/mg for juveniles and 4.93 mU/mg for adults using BApNA as substrate, 9.89 mU/mg for juveniles and 4.26 mU/mg for adults using SAPNA and 0.28 mU/mg for both life stages using Leu-p-Nan as substrate respectively.

Fig. 5: Electrophoresis of extract from *F. Subtilis* juveniles and adults. Lanes: MW – molecular weight markers - Ovoalbumin (46 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhidrase (29 kDa), trypsinogen (24 kDa) and α-lactalbumin (14.2 kDa); 1 – crude extract of *F. subtilis* juvenile; 2 – crude extract of *F. subtilis* adults.

Fig. 6: Thermal stability zymogram of digestive proteases of hepatopancreas tissue from juveniles (A) and adults (B) of *F. subtilis*. Lanes: 1-25°C; 2-35°C; 3-45°C; 4-55°C; 5-65°C; 6-75°C. White dashed arrow – extra band observed in juveniles when compared to the protease pattern observed for adults of *F. subtilis*. White arrow – thermostable band in juveniles of *F. subtilis*.

Fig. 7: Zymogram of proteolytic inhibition of hepatopancreas from juveniles (A) and adults (B) of *F. subtilis* using specific inhibitors. Lanes: C – control (without inhibitors). Inhibition: 1 – PMSF; 2 – TLCK; 3 – Benzamidine. White dashed arrows (A and B) – bands not inhibited by trypsin inhibitors. White arrow (A) – thermostable band (observed in Fig. 5) from juveniles of *F. subtilis*. Black arrow (A) – extra proteolytic band inhibited by PMSF, TLCK and benzamidine in crude extract from juveniles.

5. CAPÍTULO II – DIGESTIVE PROTEINASES FROM THE MIDGUT GLAND OF THE PINK SHRIMP *Farfantepenaeus paulensis* (Crustacea, Decapoda, Penaeidae)

ESTE ARTIGO SERÁ SUBMETIDO À REVISTA INTERNACIONAL COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY – PART B

Digestive proteases from the midgut gland of the pink shrimp Farfantepenaeus paulensis (Crustacea, Decapoda, Penaeidae)

D. S. Buarque[a,b], F. M. S. Santos[a,b], P. F. Castro[c], D. Lemos[d], L. B. Carvalho Júnior[a,b], R. S. Bezerra[a*]

[a] Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica, Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife-PE, Brazil
[b] Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Cidade Universitária, 50670-901, Recife-PE, Brazil
[c] Embrapa Meio-Norte, Caixa Postal 341, 64200-970, Parnaíba – PI, Brazil
[d] Instituto Oceanográfico, Departamento de Oceanografia Biológica, Universidade de São Paulo, São Paulo, Brazil.

Ranilson S. Bezerra.
Laboratório de Enzimologia – LABENZ, Departamento de Bioquímica, Universidade Federal De Pernambuco.
CEP 50670-420
Cidade Universitária, Recife-PE, Brazil,
Tel.: + 55-81-21268540
Fax: + 55-81-21268576
E-mail address: ransoube@uol.com.br

[*] Corresponding author. Tel.: +55 81 2126-8540; Fax: +55 81 2126-8576

Abstract

The present study describes the properties of proteases in midgut gland of the pink shrimp Farfantepenaeus paulensis**.** The enzymatic activity was characterized using unspecific and specific substrates and specific inhibitors. The effect of pH (7.2-9.0), temperature (25-65°C) and calcium (Ca^{2+}) concentration on tryptic activity were also assayed. The caseinolytic activity was detected in substrate-SDS-PAGE zymogram. Trypsin-like and chymotrypsin-like activities were observed in crude extract of *F. paulensis*. A positive correlation was detected between aminopeptidasic activity and the recommended level of essential amino acids in penaeid shrimp feed. Proteolytic activity was strongly inhibited by TLCK and benzamidine, specific trypsin inhibitors. TPCK was able to inhibit 59.34% of chymotryptic activity. The highest trypsin-like activity was detected at pH 8.0 and at 45°C. The highest chymotrypsin-like activity was obtained at alkaline pH range (7.2-9.0) and at 55°C. Trypsin-like activity was slightly activated by $CaCl_2$ and NaCl. The zymograms showed eight active bands in crude extract from midgut gland of the F. paulensis. Two thermal resistant (85°C) chymotrypsin isoforms were found. These bands were inhibited by PMSF. Studies about proteolytic enzymes of F. paulensis can be helpful for determining food digestion of this species.

Keywords: Chymotrypsin-like; Digestion; Farfantepenaeus paulensis; Midgut gland; Pink shrimp; Proteinases; Trypsin-like.

E-mail address: ransoube@uol.com.br

1. Introduction

Shrimps can relate to the environment through many stimuli, food being one of the most important. These crustaceans use the energy obtained from their diet to synthesize molecules needed to grow, survive and carry out actions such as movement, reproduction, repair of tissues and defense. To produce energy, food should be subjected to the action of enzymes, which contribute to break it down into compounds that can be absorbed by the cells in the gut (Shiau, 1998; Córdova-Murueta et al., 2003).

Digestion has been one of the most studied areas of shrimp nutrition (Fernández Gimenez et al., 2001), mainly due to the properties of the enzymes which help to determine the digestive capabilities of the shrimps (Vega-Villasante et al., 1995). Moreover, the understanding of digestive enzymes is important to the rational use of qualitative and quantitative food and feed ingredients in shrimp farming (Fernández et al., 1997; Muhlia-Almazán et al., 2003). Among the digestive enzymes from crustacean, proteases are the most studied ones (Fernández-Gimenez et al., 2002) and play a key role in assimilation of food protein (Muhlia-Almazán et al., 2003). These enzymes are also very important to digestive physiology of penaeids, mainly because it is fundamental to provide essential amino acids (Sanchez-Paz et al., 2003).

The pink shrimp Farfantepenaeus paulensis is a high-value fishery resource in Southern Brazilian coast (Peixoto et al., 2004). Moreover, this species has been considered as a potential alternative for currently cultured species *Litopenaeus vannamei* in sub-tropical and temperate areas. However, F. paulensis farming decreased due to the lack of information about feeds to sustain suitable growth of this species (Lemos et al., 2004). Thus, the aim of this work was to describe the activity of proteases from *F. paulensis* midgut gland for a better understanding of the digestion of this shrimp. This knowledge could be used as a reference for further nutritional studies of this species.

2. Materials and Methods

2.1. Materials

All reagents were of analytical grade purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

2.2. Organisms and sampling

Farfantepenaeus paulensis postlarvae were obtained from a public hatchery (Fundação Universidade do Rio Grande - FURG) and were reared at the Instituto Oceanográfico from

Universidade de São Paulo (USP) for 190 days (6-10 g live weight). During this period shrimps were raised at 24-28 ºC and fed a commercial ration with 35% crude protein. Midgut glands (40 mg/mL) were homogenized in chilled 0.15 M NaCl using a tissue homogenizer (Bodine Electric Company – Chicago, USA). Crude enzyme extracts were obtained after centrifugation at 10,000 x *g* for 25 min at 4°C and were strored at -20°C for further utilization. Total soluble protein content of each enzyme extract was determined as described by Bradford (1976) using bovine serum albumin as standard protein.

2.3. Enzyme activity assays

Total protease activity was assayed by hydrolysis of 1% azocasein dissolved in 0.1 M Tris-HCl buffer pH 8.0 (Bezerra et al., 2005). Triplicate samples of each enzyme extract (30 μL) were incubated with substrate solution (50 μL) for 60 min at 25°C. The reaction was stopped by the addition of 10% trichloroacetic acid (120 μL) and the mixture was centrifuged at 8000 x *g* for 5 min. Unhydrolyzed substrate was precipitated and supernatant (70 μL) was mixed with 1 M NaOH (130 μL). The absorbance of supernatants was measured in a microtiter plate reader (Bio-Rad 680) at 450 nm. One unit of total protease activity was expressed as the amount of enzyme required to hydrolyze azocasein and to produce a 0.001 change in absorbance per mL per min. Specific protease activity was expressed in units per mg of protein.

Trypsin activity was determined with benzoyl-DL-arginine p-nitroanilide (BAPNA), chymotrypsin activity was measured using Succinyl alanine alanine proline phenylalanine p-nitroanilide (SAPNA) and N-Succinyl-L-phenylalanine-p-nitroanilide (Suc-Phe-p-Nan) as specific substrates and leucine aminopeptidase activity was detected using leucine-p-nitroanilide (Leu-p-Nan). All substrates were dissolved in dimethylsulphoxide (DMSO) in a final concentration of 0.6 mM (Bezerra et al., 2005). The change of absorbance at 405 nm was recorded for 15 min using a microtiter reader (BIO-RAD 680). One unit (U) of activity was defined as the amount of enzyme required to produce one μmol of p-nitroaniline per minute. Specific activity was expressed as units per mg of protein.

Aminopeptidase activity was also evaluated using aminoacyl β-naphthylamide (AA-NA) as substrates. The substrates used were: AA-NA of Ala, Arg, Leu, Phe, Val, Ser, Gli, Ile, Tyr, His, Lys and Glu. The procedure adapted from Oliveira et al. (1999) was carried out by incubating 4.2 mM substrate (50 μL), 50 mM sodium phosphate buffer pH 7.0 (600 μL) and H_2O (50 μL) at 37ºC. After temperature equilibration the enzyme (50 μL) was added. After

120 minutes, the reaction was stopped by adding fresh Garnet reagent (250 µL) prepared in 0.2 M sodium acetate buffer, pH 4.2, containing 10% v/v Tween 20. Absorbance was measured at 525 nm and the amount of β-naphthylamine was determined using a standard curve of β-naphthylamine. The activity was expressed as protease milliunits per milligram of protein. One unit of enzymatic activity was defined as the amount of enzyme required to hydrolyze one µmol of β-naphthylamine per minute.

2.4. Inhibition assays

To determine the effect of specific inhibitors on enzyme activity, equal volume of crude extract and inhibitors were incubated for 30 min at 25°C and then residual activity was measured. Phenyl-methyl-sulfonyl-fluoride (PMSF) was used as inhibitor of serine proteases, tosyl-lysine chloromethyl ketone (TLCK) and benzamidine for trypsin and Tosyl-phenylalanine chloromethyl ketone (TPCK) for chymotrypsin. Volumes were then adjusted to 170 µL with 0.1 M Tris-HCl pH 8.0 and the respective substrate (BAPNA – PMSF, TLCK and benzamidine; SAPNA – PMSF and TPCK). All inhibitors were prepared in DMSO in a final concentration of 1.0 mM (Bezerra et al., 2005). The change of absorbance at 405 nm was recorded for 15 min using a microtiter reader (BIO-RAD 680).

2.5. Physical chemical characterization

To evaluate the effects of pH and temperature on proteolytic activity of crude extract of F. paulensis, enzyme extract was incubated with Tris-HCl buffer pH ranging from 7.2 to 9.0 and temperatures from 25 to 65°C, respectively. Enzyme activity was determined as described above using specific substrates.

Thermal stability was evaluated by recording enzyme activities at 25°C after their pre-incubation at temperatures ranging from 25°C to 85°C (Bezerra et al., 2005).

2.6. Effect of $CaCl_2$

The effect of $CaCl_2$ on crude tryptic activity was studied. $CaCl_2$ was added into the standard reaction assay to obtain the final concentration of 1, 5, 10, 30, 50 and 100 mM. The residual activity was determined as described above.

2.7. Zymograms

Zymograms were also carried out, in accordance with Garcia-Carreño et al. (1993). After electrophoresis, the gels were immersed in 2.5% (100 mL) Triton X-100 in 0.1 M Tris-HCl

pH 8.0 for 30 min at 4°C to remove the SDS. The Triton X-100 was removed by washing the gels three times with 100 mL of 0.1 M Tris-HCl buffer, pH 8.0. Then, the SDS and Triton X-100 free gels were incubated with 100 mL 3% casein (w/v) in 0.1 M Tris-HCl pH 8.0, for 30 min at 4°C. The temperature was raised to 25°C and maintained for 90 min to allow the digestion of casein by the active fractions. Finally, the gels were stained and destained as described above. For thermal stability zymogram, it was used the same methodology described above, except that samples were pre-incubated at temperatures ranging from 25°C to 85°C. For zymogram of enzymatic inhibition, samples were pre-incubated with serine protease and trypsin inhibitors as described above (inhibition assays). Samples incubated with proteinase inhibitors were compared with control (without inhibitors). Decrease of the intensity or evanishment of the bands indicated inhibiton of proteolytic activity, identifying the type of enzyme.

2.8. Statistics

Data (mean±standard deviation) processing was carried out using Microcal Origin 6.0. Differences among means (effect of $CaCl_2$ and NaCl) were analyzed by one-way ANOVA followed by Tukey's multicomparison test. Differences were considered significant when $p<0.05$ (Zar, 1984).

3. Results

The activity of digestive proteases in F. paulensis is showed in Table 1. Trypsin-like activity (BAPNA) was observed in F. paulensis midgut gland. It was not detected any activity using N-Succinyl-L-phenylalanine-p-nitroanilide (Suc-Phe-p-Nan), specific chymotrypsin substrate. However the chymotryptic substrate *N*-succinyl-ala-ala-pro-phe *p*-nitroanilide (SAPNA), which contains more than one amino acid, was rapidly hydrolysed when incubated with crude enzyme extract.

INSERT TABLE 1 HERE

The use of specific substrate (Leu-p-Nan) evidenced the presence of leucine aminopeptidase-like enzymes (Table 1). It was also detected a positive correlation between aminopeptidasic activity using aminoacyl β-naphthylamide as substrates and the recommended level of essential amino acids in penaeid shrimp feed (Fig. 1).

INSERT FIG. 2 HERE

The effect of four different inhibitors on the digestive proteases from F. paulensis midgut gland is presented in Table 2. Proteolytic activity on crude extract of F. paulensis using BAPNA was 37.3% inhibited by PMSF, a serine protease inhibitor. The use of TLCK and benzamidine, both synthetic trypsin inhibitors, showed a strong inhibitory effect (87.1 and 89.9%, respectively) using the same substrate. PMSF was also capable to inhibit 27.1% of chymotrypsin activity as determined by hydrolysis of SAPNA. Proteolytic activity was also inhibited by TPCK (59.3%), a specific bovine chymotrypsin inhibitor, using the same substrate.

INSERT TABLE 2 HERE

The effect of pH on enzymatic activities was evaluated in the range of 7.2 to 9.0 (Fig. 2). The highest trypsin-like activity was observed at a pH range from 8.0 to 9.0, and the maximum activity took place at 8.0 (Fig. 2A). Chymotrypsin-like enzymes also presented highest activity at pH 8.0 (Fig. 2B).

INSERT FIG. 2 HERE

The effect of temperature on proteolytic activity of crude extract from F. paulensis is showed in Fig. 3. A peak of enzyme activity at 45°C was observed in the assay with BAPNA (Fig. 3A). The activity of trypsin-like enzymes dropped when submitted to temperatures higher than 45°C. The highest chymotrypsin-like activity was observed at 55°C and higher temperatures caused a strong decrease of activity.

INSERT FIG. 3 HERE

The influence of heat treatment on trypsin-like stability is showed in Fig. 4A. Activity was higher than 75% when assayed at 25-45°C. A small residual activity was observed in temperatures higher than 45°C. At 55°C the residual activity was reduced more than 80%. Chymotrypsin-like enzymes presented low stability at temperatures as high as 55°C. It was possible to observe that about 70% of initial activity was reduced at 55°C. Chymotrypsin-like enzymes maintained toward 15% of its initial activity at 85°C (Fig. 4B).

INSERT FIG. 4 HERE

The effect of $CaCl_2$ on crude trypsin of F. paulensis was determined (Fig. 5). No significant differences ($p>0.05$) on tryptic activity were observed between 1 and 10 mM $CaCl_2$. The activity decreased after incubation at 10mM $CaCl_2$. Lower activities were recorded at 30, 50 and 100 mM respectively ($p<0.05$).

INSERT FIG. 5 HERE

Proteolytic activity present in F. paulensis midgut gland was also observed in zymograms (Fig. 6). F. paulensis presented 8 active bands at 25°C (Fig. 5; lane 1). From 35 to

55 °C the proteolytic patterns of bands were similar to those observed at 25 °C but one band was less intense at 55°C (Fig. 5; white dashed arrow). Surprisingly, two bands remained active at temperatures as high as 85°C (Fig. 5; white arrow).

INSERT FIG. 6 HERE

Protease inhibition zymogram is shown in Fig. 7. All proteolytic bands were inhibited by PMSF (Fig. 6, lane 2). TLCK (Fig. 6, lane 3) and benzamidine (Fig. 6, lane 4) were responsible for inhibiting almost all proteolytic bands. Thermoresistant bands were inhibited only by PMSF. TPCK did not affect the proteases on crude extract of F. paulensis (Fig. 6, lane 5).

INSERT FIG. 7 HERE

4. Discussion

Digestive enzyme activities may indicate differences in the digestive potential among cultured shrimps (Lemos et al., 2000). The study of the digestive potential may be a tool to assess optimal nutritional conditions for cultured penaeid species (Jones et al., 1997). Trypsin-like and chymotrypsin-like enzymes were observed in juveniles of F. paulensis. These enzymes have been reported to play an important role in carnivorous and omnivorous penaeid species (Fernández Gimenez et al., 2001; Muhlia-Almazán, García-Carreño, 2002). Generally, most enzymes from decapods are serine proteases, including trypsin and chymotrypsin, which seems to be the most important crustacean digestive enzymes (García-Carreño et al., 1994; Fernández et al., 1997). Trypsin also plays an important role in digestion through activation of zymogens of both itself and other endopeptidases. Following protein hydrolysis by endoproteases into long chained peptides, exopeptidases such as aminopeptidases further degrade protein into smaller peptides and amino acids (Natalia et al., 2004). Little information is available about aminopeptidases in shrimps, thus, in order to provide a greater understanding of these enzymes, leucine p-nitroanilide was also used.

It was also observed a higher hydrolysis of β-naphthylamide substrates containing amino acids required at higher levels in shrimp diets which are arginine (5.8% of crude protein-CP), leucine (5.4% CP) and lysine (5.3% CP) (Guillaume, 1997). These amino acids correlated with the aminopeptidasic activity using some β-naphthylamide substrates (Arg-, Leu-, Lys-, Phe- and Val-). The results corroborate the requirements reported in literature, since lysine and arginine are described as the most limiting essential amino acids in commercial shrimp feeds (Fox et al., 1995). The correlation between recommended levels of some dietary components such as lysine and arginine and high aminopeptidasic activity can

be related to efficient digestion and incorporation of these key nutrients (Lemos and Nunes, in press).

The specificity of the shrimp chymotrypsins was studied through incubation with different specific synthetic substrates. The difference between hydrolysis of SAPNA and Suc-Phe-p-Nan may have occurred because the shrimp enzymes favor substrates containing more than one amino acid (Tsai et al., 1986). They do not hydrolyse the p-nitroanilide of several *N*-acyl amino acids or dipeptides, but they hydrolyse those of acyl tripeptides or longer substrates of the correct specificity, including SAPNA (Tsai et al., 1986).

The enzymes from F. paulensis midgut gland showed properties of serine proteases. They are inhibited by PMSF, which is consistent with the presence of serine and histidine residues at the catalytic site (Sainz et al., 2004). Moreover, the most important proteases from decapod crustacean belong to the serine class and are inhibited by this inhibitor (García-Carreño et al., 1994; Lemos et al., 2002). The inhibition of three trypsin isoforms from the Pacific white shrimp Penaeus vannamei were obtained with TLCK and benzamidine when BAPNA was used as substrate (Sainz et al., 2004).

In spite of inhibiting 60% of *F. paulensis* chymotrypsin activity, TPCK was not effective on chymotrypsins from tiger prawn Penaeus monodon (Tsai et al., 1986). The use of chymostatin, an oligopeptide inhibitor of microbial origin, was more effective on enzymes of P. Monodon. It may suggests that shrimp enzymes have an active site homologous to the chymotrypsin family (Tsai et al., 1986).

Trypsin-like activity of F. paulensis was high at pH between 8 and 9 for the hydrolysis of BApNA. Jiang et al. (1991) found that optimum pH 7 to 8 was optimum for for three trypsins from P. monodon midgut gland using p- toluenesulfonyl-L-arginine methyl ester (TAME) as substrate. The highest hydrolysis ability of trypsins in other organisms such as crayfish was also obtained at pH between 7 and 8 (Dionysius et al., 1993). From pyloric caeca of sardine Sardinops sagax caerulea the best results were observed at pH 8 using BAPNA as substrate (Castillo-Yáñez et al., 2005). Crustacean proteases generally show high activities at a pH range between 5.5 and 9.0 (García-Carreño, 1992). Chymotrypsin from the gut of Daphnia magna also had optimum pH in the alkaline range (pH 8 to 10) for hydrolysis of SAPNA (Elert et al., 2004). Saborowski et al., 2004 demonstrated that chymotrypsin from gastric fluid of crab Cancer pagurus showed maximum activity in a range around pH 7 using the same substrate.

Trypsin-like enzymes from F. paulensis midgut gland presented the highest BAPNA hydrolysis at 45°C. Other organisms appeared to present maximum trypsin activity at higher

temperatures. Trypsin from the digestive tract of P. monodon exhibited maximum activity between 55 and 65 °C (Jiang et al., 1991) and in Penaeus vannamei the maximum tryptic activity occured at 50°C (Sainz et al., 2004). Maeda-Martínez et al. (2000) reported that trypsin from the crustacean Triops sp. reached maximum activity between 50 and 60°C. On the other hand, trypsin from some fish species, such as cod Gadus ogac, capelin M. villosus and anchovy E. encrasicholus and E. japonica presented similar optimum temperature (between 40 and 45°C) to trypsin-like enzymes from F. paulensis (Simpson and Haard, 1984; Martinez et al., 1988). Trypsin-like enzymes from F. paulensis were thermolabile at temperatures higher than 45°C. It suggests that most of these enzymes were denaturated at these temperatures. Trypsin from marine crab Cancer pagurus retained 70% of its initial activity after 60 min. at 50°C (Saborowski et al., 2004).

Some authors have found chymotrypsin-like activity from shrimps (Vega-Villasante et al., 1995; Fernández, et al., 1997; Lemos et al., 2000; Fernández Gimenez et al., 2001; Córdova-Murueta et al., 2004). However, information concerning the physical-chemical characterization of shrimp chymotrypsins is still scarce. Further studies are required to compare temperature effects in chymotrypsins from several shrimp species. Chymotrypsin activity from gastric fluid of crab Cancer pagurus was extinguished after incubation at 60°C for 20 min (Saborowski et al., 2004). Therefore, chymotrypsin of C. pagurus seems to be less thermostable than chymotrypsin of F. paulensis. Temperature stability of proteolytic enzymes from F. paulensis was also shown in substrate gel electrophoresis. All the bands remained active until 55°C, even though the quantitative determination of proteolytic activity was low at the same temperature. In fact, zymograms technique is more sensible than the quantitative assays using soluble substrates (Lemos et al., 2000). It is possible to observe two active bands at temperatures as high as 85°C. The responses of proteolytic enzymes from shrimps under temperature changes must be further studied.

The presence of trypsin and chymotrypsin were also determined in zymograms using synthetic inhibitors (Fig 6). However, the proteases were not inhibited by TPCK (Fig 6, lane 5). Since trypsin and chymotrypsin are serine proteases, active bands inhibited by both PMSF and TLCK were considered as trypsin. Bands inhibited only by PMSF could be an evidence of chymotrypsins (Lemos et al., 2000). Thermostable bands (observed in Fig 5) were inhibited only by PMSF (Fig 6, lane 2). TLCK (Fig 6, lane 3) and benzamidine (Fig 6, lane 4) inhibitors did not affect these bands. It may indicate that these bands are possible thermostable chymotrypsin forms. Through inhibition by PMSF Lemos et al. (2000) chymotrypsin isoforms in adults of F. paulensis.

The effect of calcium and sodium on crude extract of F. paulensis was also observed. Calcium is essential for vertebrate species; however its relevance for the activity of crustacean serine proteases need to be more investigated. On the other hand stabilizing effects of calcium were found on several proteases including serine proteases.

In conclusion, there are evidences of trypsin, chymotrypsin and aminopeptidases are present in F. paulensis midgut gland. The latter enzymatic activity showed a positive correlation with the recommended level of essential amino acids in penaeid shrimp feed. Trypsin-like enzymes were similar in physical-chemical properties from other decapod crustacean as described in the literature. Two bands with proteolytic activity at high temperatures were found in zymograms. In comparison to inhibition zymograms obtained by Lemos et al. (2000) we can conclude that these bands are possible chymotrypsins. Further studies on purification and characterization of proteases from F. paulensis midgut gland are still needed to explain the mechanism of action of these thermal resistant bands. Moreover, additional information on digestive enzymes could help to elucidate the relation between diet and digestive potential of this species. It might provide relevant information for a better understanding of feeding and nutrition of farmed F. paulensis.

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Captions to figures:

Fig. 1: Correlation between aminopeptidasic activity of F. paulensis using aminoacyl β-naphthylamide as substrates and recommended level of essential amino acids for Penaeid shrimp feed. Values are shown in mean ± Standard Deviation - SD (n=5). The activity using β-naphthylamide substrates were: Arginine – 0.21 mU/mg; Leucine – 0.078 mU/mg; Lysine – 0.17 mU/mg; Level of amino acids expressed in % of crude protein: Arginine (5.8), Leucine (5.4), Lysine (5.3), Phenylalanine (4.0), Valine (4.0). *According to Guillaume (1997).

Fig. 2: Effects of pH on proteolytic activity of F. paulensis (○) using BApNA (**A**) and SAPNA (**B**) as substrates. The values are shown in mean ± SD (n=5). The crude extract was incubated with the above substrates in different assays at the indicated pH for 15 min and the reactions were measured at 405 nm. The values were expressed as percents of the highest one (100%). The 100% values were 5.41 mU/mg using BAPNA as substrate and 13.07 mU/mg using SAPNA as substrate. BAPNA and SAPNA are specific substrates for the quantification of trypsin and chymotrypsin activities, respectively.

Fig. 3: Effects of temperature on proteolytic activity of F. paulensis (○) using BAPNA (**A**) and SAPNA (**B**) as substrates. The values are shown in mean ± SD (n=5). The crude extract was incubated with the above substrates in different assays at the indicated pH for 15 min and the reactions were measured at 405 nm. The values were expressed as percents of the highest one (100%). The 100% values were 10.635 mU/mg using BAPNA as substrate and 19.548 mU/mg using SAPNA as substrate.

Fig. 4: Effects of thermal stability on proteolytic activity of F. paulensis (○) using BAPNA (**A**) and Leu-p-Nan (**B**) as substrates. The values are shown in mean ± SD (n=5). The thermal stability was determined by assaying its activity (25°C) after pre-incubation for 30 min at the indicated temperatures. The 100% value was expressed through assay of crude extract at 25°C. The 100% values were 6.28 mU/mg using BAPNA as substrate and 13.45 mU/mg using SAPNA as substrate.

Fig. 5: Effect of $CaCl_2$ on tryptic activity of crude extract from F. paulensis. $CaCl_2$ was added in the assay to final concentration of (1-100 mM). The 100% value (6.71 mU/mg) was

expressed through assay of crude extract at 25°C. Tryptic activity was determined using BAPNA as substrate. Bars showing asterisks are significantly different ($p<0.05$).

Fig. 6: Thermal stability zymogram of digestive proteases of F. paulensis midgut gland. Lanes - proteinase activity after incubation at: 1-25°C; 2- 35°C; 3-45°C; 4-55°C; 5-75°C; 6-80°C; 7-85°C. White dashed arrow (not distinguishable) – Band less intense at 55°C. White arrow – Thermoresistant bands at 85°C.

Fig. 7: Zymogram of inhibition of proteases from crude extract of midgut gland of F. paulensis using specific inhibitors. Lanes: 1 – control (without inhibitors); 2 – PMSF; 3 – TLCK; 4 – Benzamidine; 5 - TPCK.

Tables:

Table 1: Proteolytic activity from F. paulensis midgut gland using different substrates.

Substrates	Enzymatic Activity (mU/mg protein)
Azocasein	6.49±0.20
BApNA	5.13±0.55
SAPNA	12.20±1.29
Suc-Phe-p-Nan	Not detected
Leu-p-Nan	0.20±0.02

The values are shown in mean ± SD (n=5)

BApNA: benzoyl arginine p-nitroanilide

SAPNA:Succynil-Alanine-Alanine-Proline-Phenylalanine-p-nitroanilide

Suc-Phe-p-Nan: N-Succinyl-L-Phenylalanine-p-nitroanilide

Leu-p-Nan: leucine p-nitroanilide

Table 2: Effect of specific inhibitors on Farfantepenaeus paulensis protease activity.

Inhibitors	Enzymatic Inhibition (%)
PMSF[1]	37.3 ± 0.43
PMSF[2]	27.1 ± 0.94
TLCK	86.1 ± 0.06
Benzamidine	89.9 ± 0.14
TPCK	59.3 ± 0.60

The values are shown in mean ± SD (n=5).
PMSF: Phenylmethylsulphonyl Fluoride, serine protease inhibitor; TLCK: Tosyl Lysine Chloromethyl Ketone, trypsin inhibitor;
TPCK: Tosyl Phenylalanine Chloromethylketone, chymotrypsin inhibitor.
The maximum specific proteolytic activity (100%) was 5.46 mU/mg protein using BApNA as substrate. 100% was 11.05 mU/mg protein using SAPNA as substrate.

[1] PMSF Inhibition using BapNA as substrate.

[2] PMSF inhibition using SAPNA as substrate.

Figures:

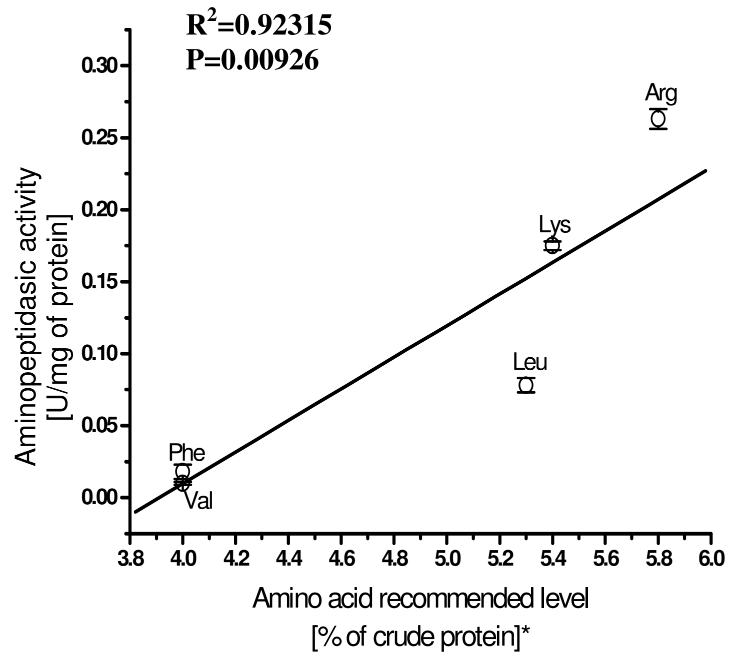
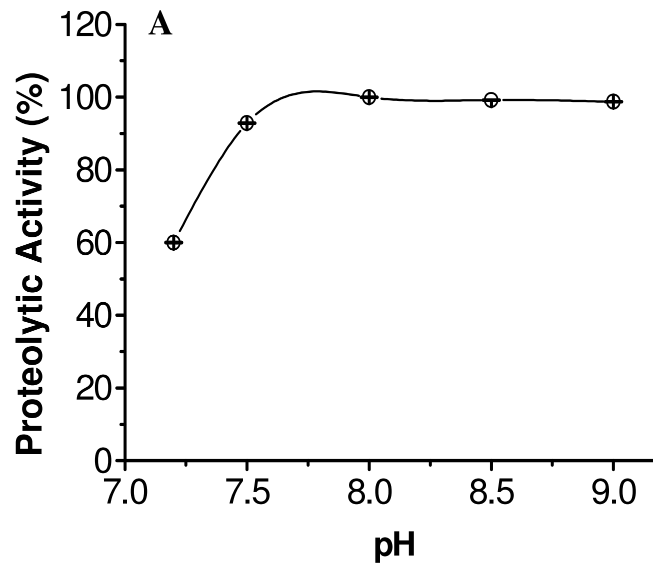
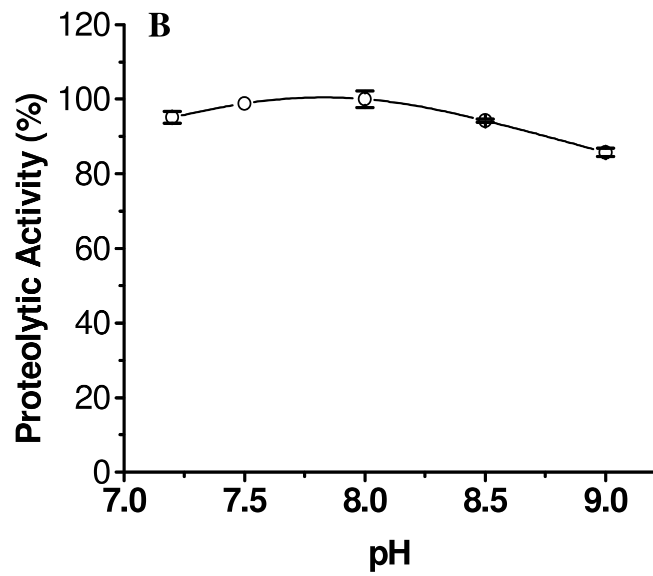


Figure 1





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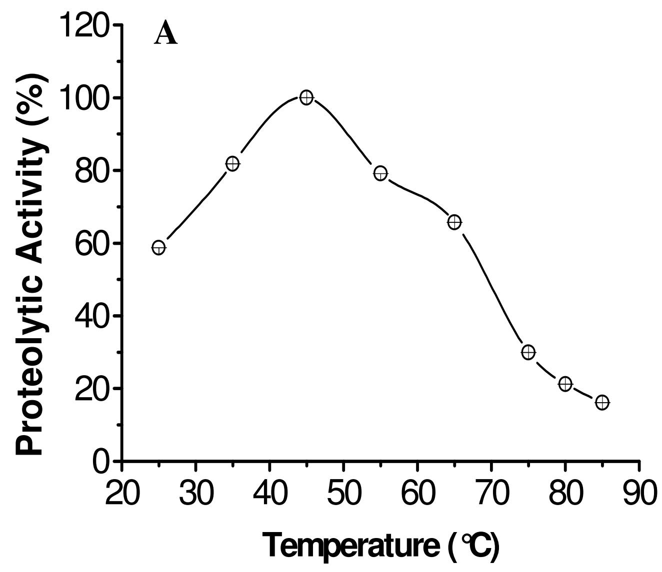

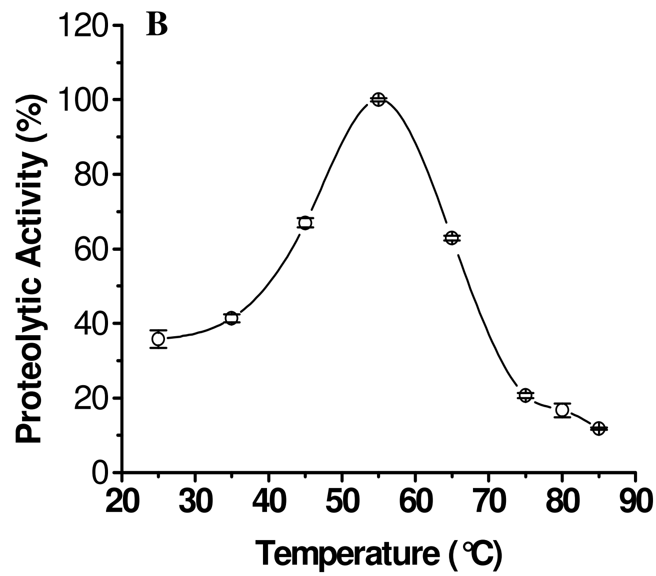

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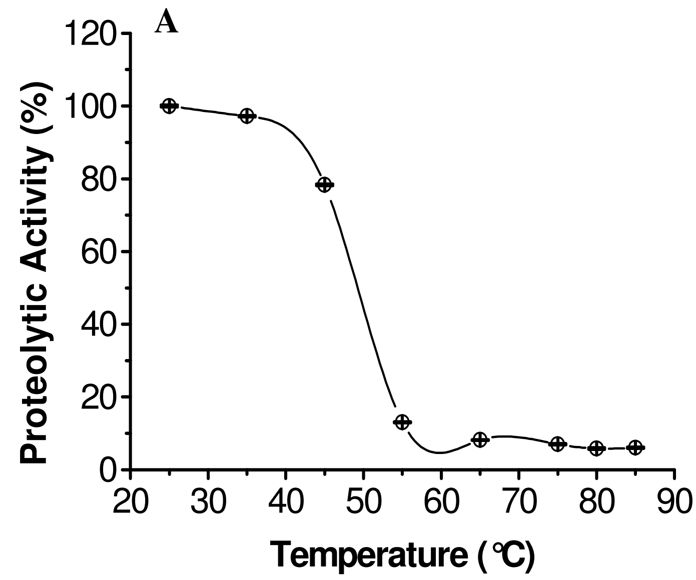

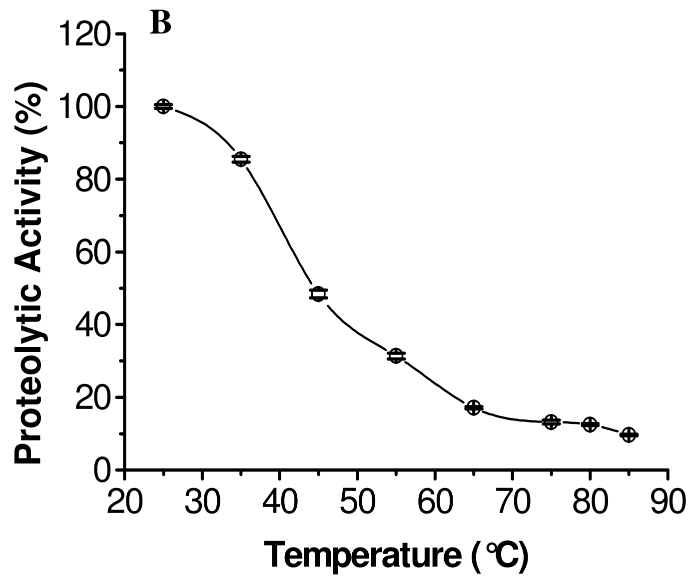

Figure 4

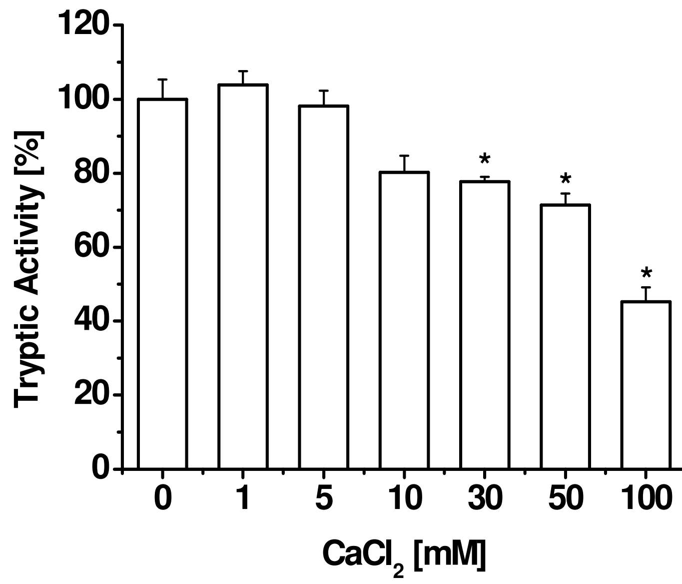

Figure 5

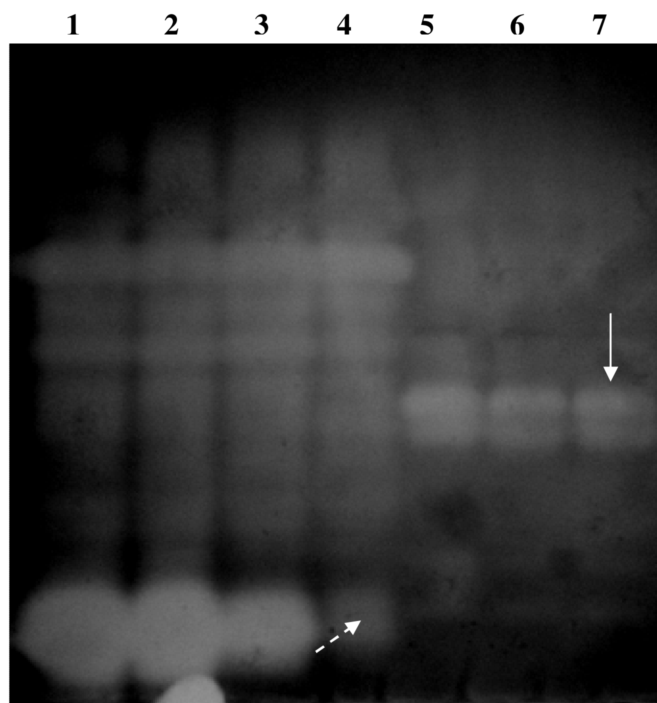

Figure 6

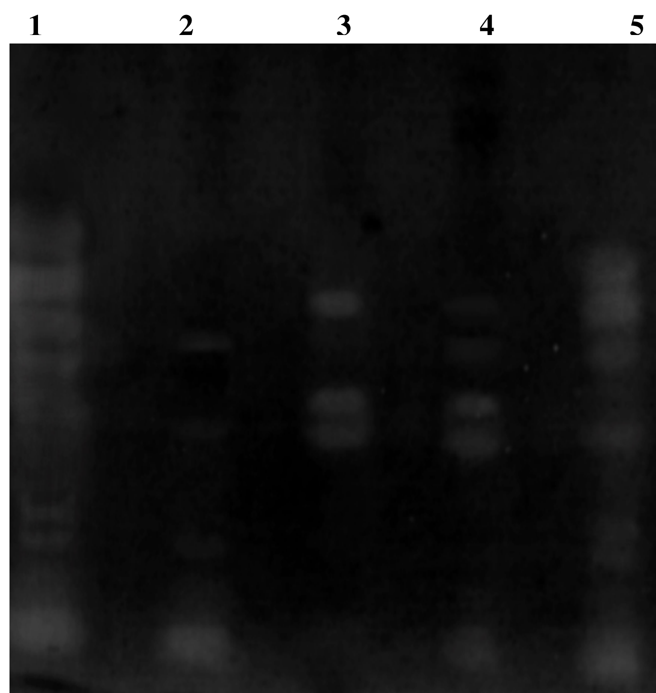

Figure 7

6. CONCLUSÕES

Os dados sugerem a presença de tripsina-símile, quimotripsina-símile e aminopeptidases nos camarões da espécie *Farfantepenaeus subtilis*. As proteases estudadas apresentaram poucas diferenças quantitativas e qualitativas em juvenis e adultos. A diferença notável entre as duas fases de vida foi uma maior atividade de quimotripsina-símile no extrato de camarões jovens, o qual também apresentou uma banda de atividade caseinolítica a mais do que em adultos, além de uma banda proteolítica estável à 65°C. A presença de tripsina-símile e quimotripsina-símile também foi detectada no hepatopâncreas da espécie *Farfantepenaeus paulensis*. Duas bandas proteolíticas com elevada estabilidade térmica (85°C) foram encontradas no extrato bruto de *F. paulensis*. Estas isoformas apresentaram características de quimotripsina, sendo inibidas por PMSF e não sendo afetadas pelos inibidores de tripsina utilizados no presente trabalho. As proteases digestivas das dua espécies estudadas mostraram propriedades físico-químicas similares às de outros crustáceos.

Informações adicionais sobre enzimas digestivas poderiam contribuir para elucidar a relação entre dieta e atividade digestiva em *F. subtilis* e *F. paulensis*. Isto pode gerar conhecimento para que haja um melhor entendimento da nutrição de camarões nativos, possibilitando, assim, o cultivo destas espécies.

7. ANEXOS

7.1. Normas da revista Aquaculture

Guide for Authors

Submission of manuscripts

Types of contribution

Original Research Papers should report the results of original research. The material should not have been previously published elsewhere, except in a preliminary form.
Review Articles can cover either narrow disciplinary subjects or broad issues requiring interdisciplinary discussion. They should provide objective critical evaluation of a defined subject. Reviews should not consist solely of a summary of published data. Evaluation of the quality of existing data, the status of knowledge, and the research required to advance knowledge of the subject are essential.
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Guide for Authors

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Lindsley, J.E., Rutter, J., 2004. Nutrient sensing and metabolic decisions. Comp. Biochem. Physiol. B 139, 543-559.
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7.3. Indicadores de produção 2006-2007

7.3.1. Resumos em congressos

Resumos na SBBq 2006

L-72 DIGESTIVE PROTEASES AND LIFE STAGES OF THE BRAZILIAN SHRIMP (*Farfentepenaus subtilis*).
BUARQUE, D.S.[1]; AMARAL, I.P.G.[1]; SANTOS, F.M.S.[1]; CASTRO, P.F.[1]; LEMOS, D.[2]; CARVALHO Jr, L.B.[1]; BEZERRA, R.S.[1]

L-74 ALKALINE PROTEASES FROM THE PINK SHRIMP (*Farfantepenaeus paulensis*)
SANTOS, F.M.S.[1]; BUARQUE, D.S.[1]; AMARAL, I.P.G.[1]; CASTRO, P.F.[1]; ESPOSITO, T. S.[1]; LEMOS, D.[2]; CARVALHO Jr, L.B.[1]; BEZERRA, R.S.[1]

L-107 THERMOSTABLE DIGESTIVE PROTEASE FROM HEPATOPANCREAS OF SEMI-TERRESTRIAL CRAB (*Cardisoma guainhumi*)
Rosso, B.U.; Espósito, T. S.; Lins, E.J.F.; Amaral, I.P.G.; Buarque, D.S.; Freitas Jr., A.C.V.; Carvalho Jr., L.B.; Bezerra, R.S.

Resumos na SBBq 2007

L - 44 DIGESTIVE PROTEASES FROM SOUTHERN SHRIMP (*Farfantepenaeus subtilis*)
Buarque, D.S.[1], Santos, F.M.S.[1], Amaral, I.P.G.[1], Castro, P.F.[1], Oliveira, S.M.[2],
Alves, K. B.[2], Carvalho Jr, L.B.[1], Bezerra, R.S.[1]

L - 45 PROPERTIES OF PROTEASES FROM THE CULTURED MARINE SHRIMP (*Farfantepenaeus subtilis*)
Santos, F.M.S.[1]; Buarque, D.S.[1]; Amaral, I.P.G.[1]; França, R. C. P.[1]; Freitas Jr, A. C. V.[1]; Castro, P.F.[1]; Carvalho Jr, L.B.[1]; Bezerra, R.S.[1]

7.3.2. Capítulo em livro

Capítulo 18

Propriedades e Aplicações Biotecnológicas das Proteases de Vísceras de Peixes

Ranilson de Souza Bezerra; Diego de Souza Buarque; Ian Porto Gurgel do Amaral; Patrícia Fernandes de Castro; Talita da Silva Espósito; Suzan Diniz dos Santos; Luiz Bezerra de Carvalho Jr.

Laboratório de Enzimologia; Departamento de Bioquímica; Universidade Federal de Pernambuco
50670-910 – Recife – PE <ransoube@uol.com.br>

Resumo

As proteases representam a mais importante classe de enzimas industriais. Estas proteínas, presentes em vísceras de peixes, um subproduto da indústria pesqueira, são usualmente subutilizadas ou simplesmente descartadas no meio ambiente. Este material pode ser uma fonte alternativa de enzimas proteolíticas, agregando valor ao processamento do pescado. Aspectos sobre purificação, fatores limitantes na produção, bem como algumas propriedades observadas em proteases de peixes tropicais e suas potenciais aplicações tecnológicas são discutidos neste texto.
Palavras-chave: proteases, tripsina, peixes tropicais, aplicações tecnológicas, indústria pesqueira

Properties and Biotechnological Applications of Proteases from Fish Viscera

Abstract

Proteases represent the most important class of industrial enzymes. These proteins, present in fish viscera, a fish industry by-product, are usually under-utilized or simply discharged in the environment. This biological material can be an outstanding source of proteolytic enzymes thus adding commercial value to the processed fish. Some aspects such as purification, production limiting factors and some properties peculiar to tropical fish proteases and its potentials in biotechnology applications are explored and discussed within this text.
Keywords: proteases, trypsin, tropical fish, biotechnological applications, fish industry

1. Introdução

Proteases digestivas representam uma importante classe de enzimas para indústria. Grande parte destas enzimas é de origem microbiana. Por outro lado, têm se buscado fontes alternativas de enzimas, principalmente devido à ampla diversidade de processos industriais, necessitando cada vez mais de proteínas com características não