Extraction, Purification and Characterization of Fish Chymotrypsin: A Review

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Abstract: Problem statement: Solid fish waste is generated from the unwanted parts of fish including heads, tails, fins, frames, offal (guts, kidney and liver) and skin. It accounts for up to 80% of material from production of surimi, 66% from production of fillet and 27% from production of headed and gutted fish. Currently, fish wastes are disposed off in land-based waste disposal systems or at sea generating toxic by-products during the decomposition process. However, fish processing waste can be used to produce commercially valuable by-products, such as chymotrypsin. **Approach:** A comperehensive review of the literature on the extraction, purification and characeterization of fish chymotrypsin was performed. **Results:** Chymotrypsin is an endopeptidase secreted by the pancreatic tissues of vertebrates and invertebrates. It has 3 different structures (chymotrypsin A, B and C) varying slightly in solubility, electrophoretic mobility, isoelectric point and cleavage specificity. Only chymotrypsin A and B are found in fish. Compared with mammal chymotrypsin, fish chymotrypsins have similar amino acid composition and molecular weights. Fish chymotrypsins have higher specific activity, especially those from cold-water fish, and low pH and temperature tolerance. The factors affecting the concentration and activity of chymotrypsin in fish are water temperature, fish species, fish age, fish weight and starvation. Chymotrypsin has application in various industries including the food industry, leather production industry chemical industry and medical industry. **Conclusion:** Extraction techniques for chymotrypsin include: ultra-filtration, ammonium sulphate fractionation precipitation or water-in-oil microemulsions. Purification can be carried out using re-crystallization and gel-filtration, ion-exchange and hydrophobic interaction chromatography. Further studies should focus on the optimization of purifiying chymotrypsin from fish processing wastes.

Key words: Chymotrypsin, trypsin, fish waste, extraction, purification, enzyme activity, ultrafiltration, fractionation, water-in-oil microemulsions, Docosahexaenoic Acid (DHA), Bovine Serum Albumin (BSA), Fish chymotrypsins

INTRODUCTION

Canada has 25% (244,000 km) of the world coastline and 16% of the world's fresh water (755,000 Km^2). In Canada, 80% of total fish landings come from the Atlantic fishery (mostly lobster, crab and shrimp and scallops) while the Pacific fishery accounts for up to 16% (mostly salmon, clams, groundfish and herring roe). The total seafood and fish income in Canada in 2007 was more than $5 billion and the exports were around $3.9 billion (AAC, 2010).

A large portion of the fish landed in Canada is processed. The most common fish processing operation includes three steps: (a) removing the viscera, (b) removing the head, tail, fins and skin and (c) removing the frame and producing fillets. Fish waste is generated from the unwanted parts of the fish which can generally be divided into two streams: (a) solid waste including heads, tails, fins, frames, offal (guts, kidney and liver) and skin and (b) wastewater from cleaning the fish and equipment. Fish processing waste can account for up to 80% of material from production of surimi, 66% from production of fillet and 27% from production of headed and gutted fish (IFC, 2011; Bechtel, 2003). In the tuna canning production, 24-30% of solid waste is generated from bones, viscera, head and skin, while another 30-35% of fish waste is generated in a liquid form from condensate and blood. Currently, fish wastes are disposed off at sea or in land-based waste disposal system (PNPPRC, 2010; AMEC, 2011). Dumping large

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volumes of wastes in water lower the level of dissolved oxygen and may generate toxic by-products during the waste decomposition process (Bechtel, 2003; Gumisiriza *et al*., 2009).

Since fish processing waste components (the heads, viscera, skin and frames) are removed in separate processes, each waste stream can be collect separately and used for the production of value added products. Potentially, fish processing wastes contain high value protein which can be reused to produce valuable by-products such as fertilizers, fishmeal and silage (IFC, 2011). Fish wastes are also known to contain highly valued fatty acids including omega-3 fatty acids such as Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA), enzymes (pepsin, trypsin, chymotrypsin), collagen and oil (Swatschek *et al*., 2002; Byun *et al*., 2003; Kim and Mendis, 2006). These valuable compounds can be used in the medical and food industries while the oil can be converted into biosel and used in the transporttion industry. Therefore, proper utilization of fish wastes can result in a tremendous commercial value (Chong *et al*., 2002).

Industrially, chymotrypsin is always produced from fresh cattle or swine pancreas and is commonly made in either tablet forms for oral consumption or as a liquid for injection. The price of chymotrypsin is related to the purity of the products. Using fish waste rather than fresh cattle or swine pancreas can dramatically lower the cost of production. There have been many studies on chymotrypsin from higher vertebrates such as cattle and swine but there is little information available on fish chymotrypsin (Einarsson *et al*., 1996). This review will focus on the recovery (extraction, purification and assay) of chymotrypsin from fish waste as a bioactive molecule and its industial and clinical uses.

FUNCTION AND STRYCTURE OF CHYMOTRYPSIN

Chymotrypsin, an endopeptidase, is a digestive enzyme existing in pancreatic tissues of vertebrates and inverbrates which is secreted into the duodenum (Geiger, 1985). Chymotrypsin hydrolyzes amides, ester and peptide ponds in protein with carboxyl side as amino acid residue at P_1 position (the first amino acid residue in the N-terminal direction from the cleaced bond). Other peptide amide bonds like tryptophanyl-, tyrosyl- and phenylalanyl- bonds are also attacked by chymotrypsin. It is also reported that leucyl and glutamyl bonds could be cleaved by chymotrypsin (Geiger, 1985; Appel, 1986). Appel (1986) indicated

that the hydrolyzing power increases according to the type of substrate in the following sequence: proteins < peptides < amides < esters < N-tyrosine esters.

Chymotrypsin has 245 amino acid residues and 5 pairs of disulfide linkages with a molecular mass around 24000. Generally, the single polypeptide molecular weight is 25-28 kDa (Haard *et al*., 2000). Chymotrypsin exists in three inactive forms (chymotrypsinogens A, B and C) in the zymogen granules (dense, membrane-bound bodies that are derived from the Golgi body of cells) of the pancreas (Smith *et al*., 1951; Geiger, 1985; Raae, 1995; Leth-Larsen *et al*., 1996). The three types of chymotrypsin (A, B, C) have been found in mammals but only two types of chymotrypsin (A and B) have been found in fish (Yang *et al*., 2009). As a one of the main digestion proteases, chymotrypsin has been widely reported in the gut of a variety of fish including: discus (*Symphysodon* sp.), carp (*Cyprinus carpto*), rainbow trout (*Oncorhyncus mykiss*), coho salmon (*O. Kistuch*), chinook salmon (*O. tshawytscha*), gilthead seabream (*Sparus awrata*) and dentex (*Dentes dentex*) (Dimes *et al*., 1994; Alarcón *et al*., 1998; Chong *et al*., 2002). Chymotrypsin concentration is 10^5 times higher in the gut than in non-gut tissue but the activity and concentration vary according to fish species and the environment in which the fish live (Elert *et al*., 2004; Parra *et al*., 2007).

Fish chymotrypsin usually has two different forms: cationic (chymotrypsin B) and anionic (chymotrypsin A) (Yang *et al*., 2009). The isoelectric points of fish chymotrypsinogens A and B are at the pHs of 9.1 and 5.2, respectively. The amino acid sequences of chymotrypsins A and B are very similar with only minor differences and the activated enzymes contain three polypeptide chains. The two isoforms of chymotrypsin (A and B) have been found in and extracted from rainbow trout (Kristjansson and Nielson, 1992), grass carp (Fong *et al*., 1998), Altantic cod (Ásgeirsson *et al*., 1993), anchovy (Heu *et al*., 1995), Monterey sardine (Castillo-Yañez *et al* 2006) and crucian carp (Yang *et al*., 2009).

The inactive form of chymotrypsin (chymotrypsinogen) can be activated by trypsin which partially cleavs it into two parts while still maintaining an S-S bond. In the enzymnogen activation process, chymotrypsinogen goes through different forms of intermediate products (α-chymotrypsin, γ-chymotrypsin, δ-chymotrypsin, π-chymotrypsin) before forming chymotrypsin A. However, the type of intermediate products generated depend on trypsin concentration and reaction velocities as shown in Fig. 1 (Geiger *et al*., 1985).
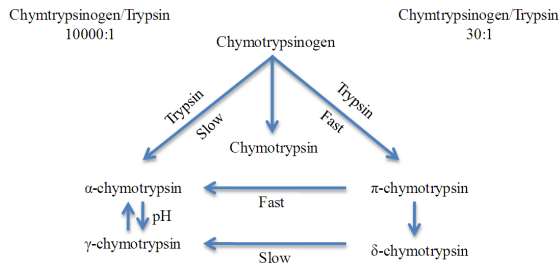
Fig. 1: Activation of chymotrypsinogen. Different chymotrypsins result as reaction products, depending on relative concentrations and reaction velocities (Appel, 1986)
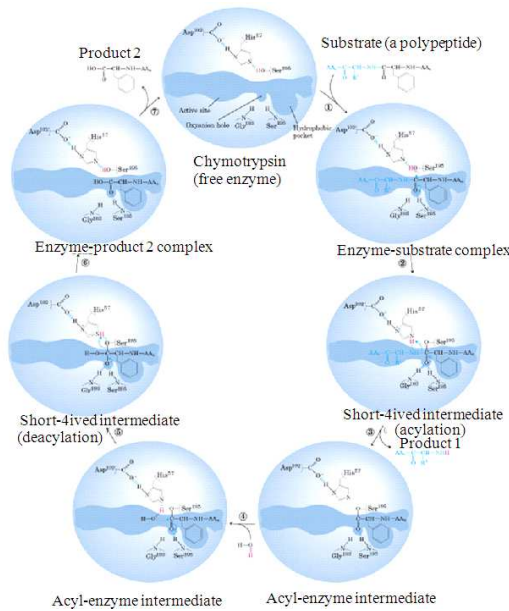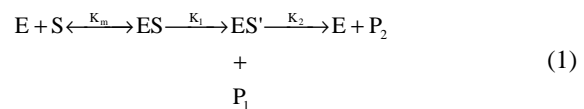

Fig. 2: The catalytic mechanism of chymotrypsin (Nelson *et al*., 2008)

CHYMOTRYPSIN SPECIFICITY AND MECHANISM OF HYDROLYSIS

Specificity: Chymotrypsin hydrolyzes peptide bonds with various α-amino acid carbonyl groups and attacks larger nonpolar aromatic groups such as tyrosine,phenylalanine and tryotophan (Folk *et al*., 1970; Appel *et al*., Galvão *et al*., 2001). It also attacks nonpolar groups like leucine but the reaction is slower. Hudáky *et al*. (1999) reported that chymotrypsins A and B have similar specificities in hydrolyzing peptides with Phe, Trp, Leu and Tyr residues. Raae *et al*. (1995) found specificity differences between two types of chymotrypsins (ChT1 and ChT2) isolated from cod.

They pointed out that ChT1 can hydrolyze 8 peptide bonds [Leu(6)-Cys(7), Leu(15)-Tyr(16), Tyr(16)-Leu(17), Arg(22)-Gly(23), Phe(25)-Tyr(26),Phe(1)-Val(2), Leu(17)-Val(18) and Tyr(26)-Thr(27)] while ChT2 has only 4 major cleavage sites [Tyr(16)-Leu(17), Arg(22)-Gly(23), Phe(25)-Tyr(26) and Tyr(26)-Thr(27)] and 2 minor cleavage sites which are peptide bonds between [(Phe(1)-Val(2) and the Phe(24)-Phe(25)].The difference in the hydrolysis specificity of chymotrypsin can be used to determine the enzyme activity utilizing specific substrates (Geiger, 1985; Srinivas *et al*., 2009). However, most substrates used as chymotrypsin substrates can also be hydrolyzed by other proteases like trypsin. Because of this, the use of highly sensitive substrates such as Suc-(Ala)$_2$-Pro-Phe-4-NA and Suc-phe-4-NA has been recommended (Geiger, 1985).

Mechanism of catalyzed hydrolysis: The process of chymotrypsin-catalyzed hydrolysis (Fig. 2) can be divided into two steps in which acyl-enzyme plays a role as intermediates (Parker and Wang, 1968; Kallies *et al*., 1996). In the first step, the substrate binds with the pocket structure of chymotrypsin. The side chain Ser-195 and His-57 attacks the substrate (hydrolyzes it) to form an intermediate. In the second step, solvent water cleaves the acyl-enzyme (ester intermediate) to form carboxylic acid and reforms the enzyme (Kallies *et al*., 1996). The overall reaction is described by the following equation (Bender and Ferbnj, 1964).

$$E + S \xrightleftharpoons{K_m} ES \xrightarrow{K_1} ES' \xrightarrow{K_2} E + P_2 \atop + \atop P_1 \qquad (1)$$

Where:

E = The enzyme
ES = The enzyme-substrate complex
ES' = The intermediate (acyl-enzyme) which is found in every case
K_1, K_2 = The first order rate constants for the acylation and deacylation steps
K_m = The Michaelis constant
P_1, P_2 = Products (proteins)

The reaction is a pH dependent and the optimal pH is approximately 7. Parker and Wang (1968) reported that K_2 dramatically increased with an increase in pH when the pH was lower than 7 but once the pH is greater than 7 no significant differences were observed. Similar observations were reported by other researchers (Fersht and Requena, 1971; Lucas *et al*., 1973; Leslie and Wang, 1968).

Table 1: Effect of various proteinase inhibitors and metal ions on chymotrypsins (Castillo-Yañez *et al.*, 2009; Yang *et al.*, 2009)

Chemical	Concentration (mM)	Residual enzyme activity (%)				
		Chy A	Chy B	Chy I	Chy II	Bovine chy
None		100.0	100.0	100	100	100
TPCK	0.05	35.6	17.5	56	60	53
Chymostatin	0.01	0.0	0.0	-	-	-
Benzamidine	5.00	60.3	62.5	103	71	117
Pefabloc SC	1.00	1.2	1.1	-	-	-
PMSF	1.00	0.4	0.3	1	2	4
SBTI	0.50	-	-	0.5	0	17
Pepstatin	0.03	95.9	92.3	-	-	-
EDTA	1.00	99.0	97.0	98	65	96
	10.00	98.0	100.0	-	-	-
CaCl₂	1.00	107.0	101.0	-	-	-
	5.00	115.0	103.0	-	-	-
MgCl₂	1.00	102.0	105.0	-	-	-
	5.00	111.0	110.0	-	-	-
MnCl₂	1.00	98.5	51.1	-	-	-
	5.00	85.3	48.9	-	-	-
CdCl₂	1.00	86.8	59.1	-	-	-
	5.00	76.8	49.7	-	-	-
FeSO₄	1.00	26.4	27.8	-	-	-
	5.00	14.6	21.4	-	-	-
CuCl₂	1.00	19.8	21.1	-	-	-
	5.00	0.0	2.5	-	-	-

Chy I and II: 2 types of chymotrypsin isolated from Monterey sardine. TPCK: tosyl-phenylalanine chloromethyl-ketone. PMSF: phenyl-methyl-sulphonyl-fluoride. SBTI: soybean trypsiin inhibitor. -: not tested

Inhibitors: Synthetic and natural inhibitors can decrease the ability of chymotrypsin to hydrolyze proteins or peptides by forming enzyme-inhibitor structures (Guha and Sinha, 1984; Makkar *et al.*, 2007). Natural chymotrypsin inhibitors are found in plants such as phaseolus lunatus, black gram, macrotyloma axillare, solanum tuberosum, pigeon pea, chick pea, erythrina caffra and patato (Makkar *et al.*, 2007) or animal sources such as egg whites (Guha and Sinha, 1984). Natural inhibitors have a wide tolerance range of pH but are heat sensitive (Guha and Sinha, 1984; Makkar *et al.*, 2007). Synthetic chymotrypsin inhibitors such as N-Toluenesulfonyle-Phenylalanine Chloromethyl Ketone (TPCK), N-carbobenzoxy-(Gly)2-Phenylalanine Chloromethyl Ketone (ZGGPCK), N-carbobenzoxy -L-phenylalanine Chloromethyl Ketone (ZPCK) and chymostatin are chymotrypsin specific and can dramatically inhibit the catalyzing power of chymotrypsin (Heu *et al.*, 1995; Sabapathy and Teo., 1995; Tokumoto *et al.*, 1999; Santigosa *et al.*, 2008).The effects of inhibitors on the chymotrypsin activity are shown in Table 1. Serine proteinase inhibitors such as Phenylmethanesulfonyl Fluoride (PMSF) and 4-(2-Aminoethyl)-benzenesulfonyl fluoreide (Pefabloc SC) effectively inhibit the activity of chymotrypsin (Elert *et al.*, 2004; Yang *et al.*, 2009) while asparatic proteinase inhibitors such as pepstatin and the metalloproteinase inhibitor EDTA have slight influences on chymotrypsin (Yang *et al.*, 2009; Heu *et al.*, 1995; Castillo-Yañez *et al.*, 2006). Metal ions such as Mn^{2+},

Cd^{2+}, Cu^{2+}, Fe^{2+} can inhibit chymotrypsin but their inhibition abilities are different (Castillo-Yañez *et al.*, 2006; Yang *et al.*, 2009).

CHYMOTRYPSIN ACTIVITY AND STABILITY

The pH, temperature, source and storage are the most significant factors influencing the activity and stability of chymotrypsin.

pH: The pH range of chymotrypsin activity is 7.5-9.0 (Kristjansson *et al.*, 1992). Castillo-Yañez (2009) studied the effect of pH on activity of sardine and bovine chymotrypsins and found that the activity of chymotrypsin was significantly inhibited when the pH was <6 and the enzyme was denatured at a pH of 4 but was stable at a pH of 8 (Fig. 3). Similar results were obtained by Tsai (1986) for chymotrypsin isolated from *P. monodon* hepatopancreas. Sabapathy and Teo (1999) found the optimal activity of rabbitfish chymotrypsin to be within the pH range of 7.5-8.5. Lam *et al.* (1999) reported that the optimal activities for 2 types (CTR1 and CTR2) of chymotrypsin extracted from *Locusta migratoria* were in the pH ranges of 8-10 and 8-11, respectively. Similar results were reported for *European sea bass* (Alliot *et al.*, 1973), *Dover sole* (Clark *et al.*, 1985), *skipjack* (Joakimsson and Nagayama, 1990), *Siganus canaliculatus* (Sabapathy and Teo, 1995), anchovy (Heu *et al.*, 1995) and Bluefin tuna (Parra *et al.*, 2007).
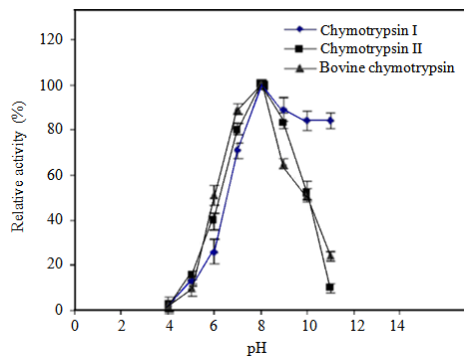
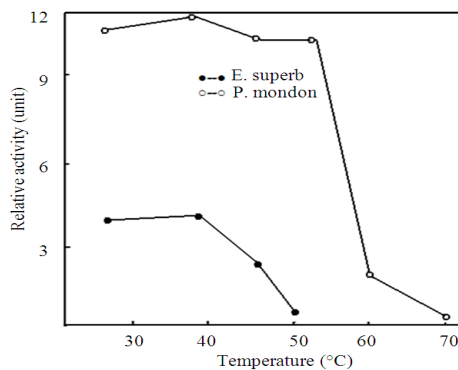Fig. 3: Effect of pH on Chymotrypsin activity at 25°C (Castillo-Yañez *et al*., 2009)

Fig. 4: Thermostability of chymotrypsin activities from the hepatopancreases (Tsai *et al*., 1986)

Temperature: Temperature has a significant effect on the stability of chymotrypsin. Tsai *et al*. (1986) studied the thermal stability of one type of chymotrypsin isolated from P. monodon and E. superb and found denaturation of the enzymes when the temperature was above 56°C and 45°C, respectively (Fig. 4). Similar results were reported by Heu *et al*. (1995) for a chymotrypsin extracted from anchovy. Sabapathy and Teo (1995) reported an optimal temperature range of 45-55°C for chymotrypsin extracted from rabbitfish and observed denaturation of the enzyme at a temperature of 60°C. Similar changes were detected for chymotrypsins extracted from Siganus canaliculatus (Sabapathy and Teo, 1995), anchovy (Heu *et al*., 1995), bluefin tuna (Parra *et al*., 2007) and Monterey sardine (Castillo-Yañez *et al*., 2006; Castillo-Yañez *et al*., 2009).

Source: The enzyme activities differ depending on the source from which the enzymes were isolated. Chymotrypsins extracted from marine fish such as the Altantic cod (Gadus morhua), herring (Clupeas harengus), capelin (Mallotus villosus), rainbow trout

(Oncorhynchus mykiss), anchovy (Engraulis japonica) and spiny dogfish (Squalus acanthias) contain higher enzyme activity but are less heat stable than mammalian chymotrypsins (Kristjansson and Nielson, 1992). Carp (warmwater fish) chymotrypsin has similar physical and kinetic properties as mammal chymotrypsins (Cohen *et al*., 1981a; Cohen *et al*., 1981b). K_{cat}/K_{m}, a ratio considered as a measure of enzyme efficiency (K_{cat}: constant for rate-limiting step; K_{m}: The Michaelis constant). The K_{cat}/K_{m} of carp is 15 while bovine, anchovy, rainbow trout and monterey sardine chymotrypsins I and II are 21, 165, 62.8, 251 and 100 (Heu *et al*., 1995; Castillo-Yañez *et al*., 2006; Castillo-Yañez *et al*., 2009).

Storage: Folk (1970) stated that dry, purified chymotrypsin powder can maintain its activity up to 4 years under frozen conditions without significant losses. He also claimed that chymotrypsin in water or Tris buffer (pH = 6-8.5) solution can be stored up to several weeks in a sterile environment. However, under acidic pH conditions, the enzyme activity is rapidly lost. Tsai *et al*. (1986) and Castillo-Yañez *et al*. (2009) reported a 12% loss in chymotrypsin activity in 20 min at a pH of 3. Castillo-Yañez *et al*. (2006) evaluated chymotrypsin storage in different buffers under various pH conditions and found that the activity is most stable under a pH of 8.0 and in the pH range of 5.0-9.5 no significant differences were observed. No information on the effect of light during storage was found in the literature.

DIFFERENCES BETWEEN FISH AND ANIMAL CHYMOTRYPSINS

Chymotrypsins isolated from fish and animals such as bovine and swine have four major differences: pH stability, temperature stability, enzyme activity and molecular weight However, their amino acid compositions are still similar (Heu *et al*., 1995; Castillo-Yañez *et al*., 2009).

pH Stability: The optimum pH for fish chymotrypsin and mammal chymotrypsin are quite similar (approximately 8.0). Castillo-Yañez *et al*. (2009)claimed that two types of chymotrypsin isolated from Monterey sardines had an optimum pH similar to that of bovine chymotrypsin (8.0). Sabapathy and Teo (1995) found that the optimum pH for rabbitfish chymotrypsin was 8.0. Castillo-Yañez *et al*. (2009) reported that fish chymotrypsin was stable only under alkaline pH condition but animal chymotrypsin was stable at both alkaline and acidic pH. For instance, bovine chymotrypsin was found to be stable at a pH of 3.0 (Wilcox, 1970; Bender and Killheffer, 1973) and rat

chymotrypsin was found to be stable in the pH range of 2.0-4.0 (Kumar and Pattabirman, 1996). However, no activity was found for two chymotrypsins isolated from Monterey sardine at a pH <4.0 (Castillo-Yañez *et al.*, 2009) and cod chymotrypsin was unstable at a pH <5.0 (Heu *et al.*, 1995). Castillo-Yañez *et al.* (2006) reported that sardine chymotrypsin was more stable in basic environments than acidic ones. Chymotrypsin from tuna was stable within the pH range of 7-10 and only 20% residual activity was found within the pH range of 3-5 (Parra *et al.*, 2007).

Temperature stability: The chymotrypsins isolated from marine fish living in cold environments (carp, rainbow trout, menhaden, anchovy, sardine and Atlantic cod) have higher catalytic activity at low temperature conditions and are more cold stable (but less heat stable) than mammal chymotrypsins (Kristjansson and Nielson, 1992; Ásgeirsson and Bjarnason, 1993; Heu *et al.*, 1995; Castillo-Yañez *et al.*, 2006; Cohen *et al.*, 1981a; Martinez *et al.*, 1988; Raae *et al.*, 1989). Castillo-Yañez *et al.* (2009) stated that the optimum temperature for sardine chymotrypsin was 45°C, ten degrees lower than bovine chymotrypsin. Castillo-Yañez *et al* (2006) reported that when sardine chymotrypsin was incubated at 55°C for 15 min no residual activity was observed but bovine chymotrypsin was inactivated when the temperature was >60°C. Ásgeirsson *et al.* (1995) reported that calf chymotrypsin was stable at 50°C while cod chymotrypsin denatured at a temperature of 40°C. Kumar and Pattabiraman (1996) reported that rat chymotrypsin was stable at 50°C for 1 h but 92% of activity was lost at 60°C in 5 min.

Table 2: Amino acid composition of Atlantic cod and anchovy chymotrypsin compared with chymotrypsin from mammal species

Amimo Acid	Atlantic cod[a]		Anchovy[b]	Bovine[c]		Dog[d] B	Human[e] B	Rat[f] B
	A	B		A	B			
Ala	21	20	12	22	23	21	22	19
Arg	9	9	9	4	5	4	6	3
Asn	14	16	-	14	7	9	9	8
Asp	9	9	25	9	13	12	15	14
Cys	10	10	5	10	10	10	10	10
Gln	10	11	-	10	10	13	9	12
Glu	9	9	21	5	8	5	6	9
Gly	25	22	17	23	23	24	23	23
His	6	6	10	2	2	4	3	2
Ile	10	13	10	10	9	11	12	11
Leu	18	15	14	19	19	20	19	6
Lys	8	7	6	14	11	13	15	14
Met	6	6	2	2	4	3	2	4
Phe	6	4	4	6	7	9	7	8
Pro	11	14	15	9	13	13	13	14
Ser	18	24	21	28	22	21	23	21
The	18	15	21	23	23	21	17	20
Trp	8	9	6	8	8	8	8	8
Tyr	5	4	3	4	3	1	2	2
Val	24	24	19	23	25	23	24	27
Total	245	247	234	245	245	245	245	245

[a]: Guðmundsdóttir *et al.* (1994) [b]: Heu *et al.* (1995) [c]: Bell *et al.* (1984) [d]: Pinsky *et al.* (1983) [e]: Smillie *et al.* (1968) [f]: Tomita *et al.* (1989)

Activity: It is well known that the catalytic activities of fish chymotrypsins are higher than warmblooded animal chymotrypsin under optimal condition (Kristjansson and Nielson, 1992; Castillo-Yañez *et al.*, 2009; Cohen *et al.*, 1981a; Cohen *et al.*, 1981b). Ásgeirsson and Bjarnason (1993) found that the rate constant (K_{cat}/K_m) of cod chymotrypsin was 3-4 fold higher than bovine chymotrypsin. Fish chymotrypsin not only maintains higher activity than mammal chymotrypsin but also has higher specific activity than mammal chymotrypsin (Heu *et al.*, 1995; Castillo-Yañez, *et al.*, 2006; Raae and Walther, 1989). Castillo-Yañez *et al.* (2009) reported that K_{cat}/K_m was 21 for bovine chymotrypsin compared to 15, 165, 62.8, 251 and 100 for chymotrypsins from crap, anchovy, rainbow trout and Monterey sardine, respectively.

Molecular weight: The molecular weight of chymotrypsin isolated from various fish ranges from 22,000-30,000 Da and the amino acid composition is slightly different among chymotrypsins. The amino acid compositions of fish and animal chymotrypsins are shown in Table 2. There are 245 amino acid residues in chymotrypsin of all mammals (bovine, dog, human and rat) and the sequences are very similar. Numbers of amino acid residues in fish chymotrypsins vary slightly. For instance, the numbers of residues in anchovy chymotrypsin and cod chymotrypsin B are 234 and 247, respectively. The sequence of Atlantic cod chymotrypsins A and B are very close but the differences are significant between the two species. Two Amino acids Asn and Gln which exit in Atlantic cod chymotrypsin are absent in anchovy chymotrypsin.

FACTORS INFLUENCING THE CONCENTRATION AND ACTIVITY OF FISH CHYMOTRYPSIN

Trypsin is an important protease which can activate chymotrypsin during the digestive process. Therefore, the factors that influence the activity of trypsin will affect the activity of chymotrypsin. Both internal factors (trypsin phenotypes and fish life stage) and external factors (water temperature and fish starvation, feeding and nutrient condition) affect the activity of chymotrypsin.

Water temperature: Haard (1995) reported that water temperature affects the chymotrypsin activity in fish intestine. Rungruangsak-Torrissen *et al.* (2006) studied the relationship between water temperature and chymotrypsin activity and found the chymotrypsin activity of Atlantic salmon to be significantly higher at 6°C than at 10°C (Kofuji *et al.*, 2005). It is reported that approximately 95% of the volume of the Atlantic Ocean is colder than 5°C (Hultin, 1980). Therefore, using

Atlantic fish wastes for production of chymotrypsin will offer higher chymotrypsin concentrations because the high latitude and low water temperature.

Fish species: Fish species has a significant influence on the concentration and activity of chymotrypsin. Based on their food habit, fish are separated into carnivores, omnivores and herbivores. Fish food habit would affect their gut weight to body weight ratio and the protealytic activity (Chakrabarti *et al*., 1995; Hidalgo *et al*., 1999). Hofer and Schiemer (1981) reported that proteolytic activity relates to different feeding habits. Although carnivorous species have smaller guts compared to herbivore species, they have higher activity. Chakrabarti *et al*. (1995) and Hidalgo *et al*. (1999) reported similar findings.

Fish age and mass: Fish age will influence the chymotrypsin concentration and activity in fish. The chymotrypsin activity is very low (0.085Umg/protein) when the fish hatches, increases very slowly in the first 2-3 weeks, then dramatically increases in the next three days and finally increases at a constant rate (Chakrabarti *et al*., 1995; Cuvier-Peres and Kestemont, 2001; Chakrabarti *et al*., 2006). The total chymotrypsin activity is positively related to fish mass which is also correlated to fish age (Dabrowski and Glogowski, 1976; Rungruangsak-Torrissen *et al*., 2006). Practically, in chymotrypsin manufacturing, fish weight can be a parameter in selecting raw material.

Starvation: Chymotrypsin will accumulate in the pancreas tissue when there is no food in the gut. Einarsson *et al*. (1996) reported that chymotrypsin activity of Atlantic salmon (in pyloriccaeca/pancreas) increased in the first few days but was reduced in the following days. This was explained by the reduced secretion of chymotrypsin from the pancreatic tissue to the intestinal digestion system and the accumulation in the pancreatic tissue. During starvation the level of chymotrypsin in intestinal keeps decreasing. Dabrowski and Glogowski (1976) reported that the starving rainbow trout showed higher chymotrypsin activity in intestine than the fed fish. Therefore, the effect of starvation effect on chymotrypsin activity will depend on the type of fish (Chakrabarti *et al*., 1995; Dabrowski and Glogowski, 1976).

INDUSTRIAL APPLICATIONS OF CHYMOTRYPSIN

Chymotrypsin has been used extensively in the food processing, leather production, chemical and medical industries.

Food industry: Chymotrypsin can be used to improve the food nutritional value of proteins and to lower the protein denaturation temperature and cleavage specificity (Yamashita *et al*., 1976; Haard, 1992). It is used in meat tenderization, fermentation, protein hydrolysate production and bone protein removal (Haard, 1998). In the dairy industry, chymotrypsin is used together with trypsin to hydrolyze casein, the main protein content in milk. Hydrolyzed by chymotrypsin, casein functional properties (such as antioxidant activity, angiotensin converting enzyme inhibition and antibacterial ability) are improved (Srinivas and Parksh, 2009). Chymotrypsin is used as an additive with trypsin to control hydrolysis of cheese whey proteins and β-lactoglobulin in the cheese production industry (Galvão *et al*., 2001).

Leather production: In the leather industry, chymotrypsin has been used in production processes including dehairing, bating and soaking. In the bating process, the specific catalystic ability of chymotrypsin has been used in hydrolyzing nonleather-forming proteins such as mucoids, globulin and albumins in raw materials. It can also help tanning materials and other chemicals to penetrate into fibers in order to obtain the desired texture (Kamini *et al*., 1999; Nathalie *et al*., 2004; Sandhya *et al*., 2005; Cera, 2008).

Chemical industry: In the detergent industry, chymotrypsin is added into laundry detergent or dish detergents to enhance the decontamination ability of the detergent. The enzyme remains stable in both ionic and non-ionic surfactants and maintains around 80% of its catalyzing ability after 1 h of incubation with chemical detergent (Espósito *et al*., 2009). Due to the specificity of chymotrypsin on proteins and peptides, it can be used to break down proteinaceous contaminatnts (blood and foods) on cloth efficiently. Another advantage of using chymotrypsin is its ability to work under mild conditions such as low water temperature or natural pH environments which may lower the damage to cloth and body (Kamini *et al*., 1999). A US patent for detergent (Patent Number: 5, 269, 959) lists chymotrypsin as a Liquid Deep Cleaning Detergent Composition (Schreibman *et al*., 1993). Another US patent (Patent Number: 20090281010) uses chymotrypsin in an eco-friendly laundry detergent composition comprising natural essence (Carter *et al*., 2009). Gupta *et al*. (2002) reported that at least 25% of extracted proteases is used as additives in laundry detergents every year. Espósito (2009) isolated chymotrypsin from fish processing waste and added it to laundry detergent to demonstrate high temperature tolerance and high enzyme stability (only

15% enzyme activity was lost after 30 min incubation less than 60°C).

Medical application: Chymotrypsin has been used in the treatment of dyspepsia and anorexia, cataract extraction, infertility and snakebite and as an anti-inflammation drug. It has been applied in several ways including: oral, local injection and atomized inhalation.

Dyspepsia and anorexia: Dyspepsia and anorexia are defined as disturbed, difficult or painful digestion. The incomplete protein digestion is dangerous because it may result in allergies or even production of toxic materials by bacterial breakdown of the incompletely digested protein. Chymotrypsin can contribute to proper digestion of proteins (Sims, 2001). Normally, people do not need to supplement with additional proteolytic enzymes because the body can produce them but pancreatic insufficiency does occur because of chemotherapy, physical injuries, chronic stress, cystic fibrosis and acute pancreatitis so extra chymotrypsin supplication may be required (Sims, 2001).

Anti-Inflammation and prevention of wound infection: Chymotrypsin has been reported to have significant inhibition against the early stage of inflammation. It is widely used both orally and by injection as a non-steroidal anti-inflammatory drug (NSAID) in the treatment of athletic injuries, wound infections, sciatica and hand trauma (Seppa, 1980). Chymotrypsin dissolves soft fibrin and cleans the proteinecious debris at inflammation sites (Swamy and Patil, 2008). Latha *et al.* (1997) used a trpsin-chymotrypsin combination in the ratio of 6:1 as an anti-inflammation agent to treat burn patients and found superior results compared to the use of trypsin alone.

Cataract extraction: Alpha chymotrypsin is widely used to separate cataractous lens from the zonular attachment sites (Hill, 1960; Rhee *et al.*, 1999; Rich *et al.*, 1974). Chymotrypsin decreases the manipulating force required during the surgery to make it easy to remove the cataract which guarantee a higher success rates in surgery (O'Malley and Straatsma, 1961). It hass been shown that α-chymotrypsin specially attacks certain components of the zonules and does not cause any damage to other tissues in the eye (Hill, 1960; Yorston and Kiku, 2000) claimed that chymotrypsin was the fastest and cheapest way to treat the cataract. Using α-chymotrypsin during the cataract surgery can help avoid significant intraocular pressure (IOP). Rapidly raised IOP may cause pain and comeal oedema to patients (Rich *et al.*, 1974; Passo *et al.*, 1985).

Infertility: α-chymotrypsin can play a significant role in the process of semen liquefaction. It can shorten the semen liquefaction time and make it less viscous without influencing sperm motility. Combined with intrauterine insemination, α-chymotrypsin has been used in infertility treatment experiments on 38 infertile patients and 23% of them became pregnant. Zhang (2005) claimed that twice-weekly injection of 5mg chymotrypsin in 61 cases of infertile patients led to a cure rate of 83.6%. The advantages of using chymotrypsin are shorter and more effective treatment of infertility at a lower cost.

Snakebite: Chymotrypsin has been used to treat patients with snakebite with good results (Omogbai *et al.*, 2003). Zhang *et al.* (2005) studied the effect of local chymotrypsin injection on patients with bites from Chinese cobra and the results showed that it had less primeval effect than local injection of antivenom but better effects than other snakebite drugs. The toxins of snakes usually contain neurotoxin and cytotoxin which can change the permeability of cell membranes and the concentration of ions like Na^+, K^+ or Ca^{2+} causing cell death. The low amount of proteases kuronidase in snake toxin can accelerate this process (Zhang *et al.*, 2005). Local chymotrypsin injection can slow down or stop this process because chymotrypsin can cleave the snake toxin protein. The hydrolyzed protein loses its toxic properties and becomes harmless to human (Zhang *et al.*, 2005). Omogbai (2003) reported that the use of chymotrypsin to treat snakebite patients was shown effect especially for those patients with tissue inflammation and edema.

EXTRACTION AND PURIFICATION OF CHYMOTRYPSIN

Generally, the initial recovery steps of chymotrypsinogen (the inactivated zymogen precursor of chymotrypsin) are: (a) extraction which includes preparation of crude material, homogenization using a buffer to extract crude chymotrypsin (chymotrypsingen) from the prepared material and centrifugation to separate the crude chymotrypsin and (b) precipitation or fractionation to collect chymotrypsingen. The processes that can be used extract chymotrypsin from fish waste are shown in Fig. 5-7.

Extraction: The fish stomach and intestines are removed from fish and separated as soon as the fish has been killed and washed with cold water or isotonical saline solution to get rid of blood in the tissue. The inhibitors in blood can reduce chymotrypsin activity (Chong *et al.*, 2001; Boeris *et al.*, 2009).
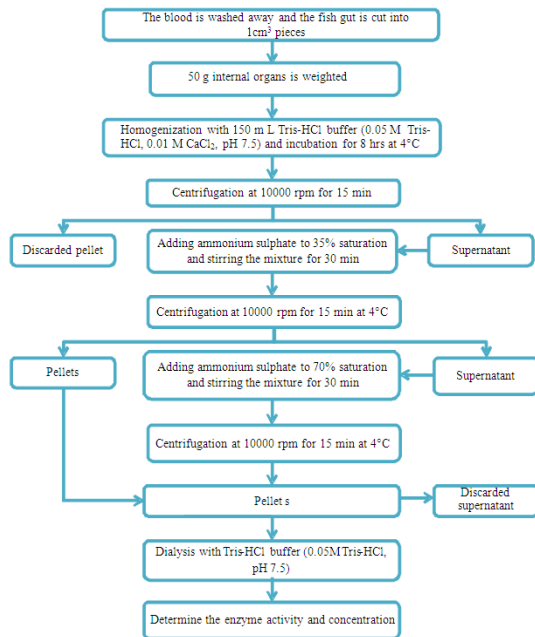
Fig. 5: Extraction of chymotrypsin from fish waste using ammonium sulphate method
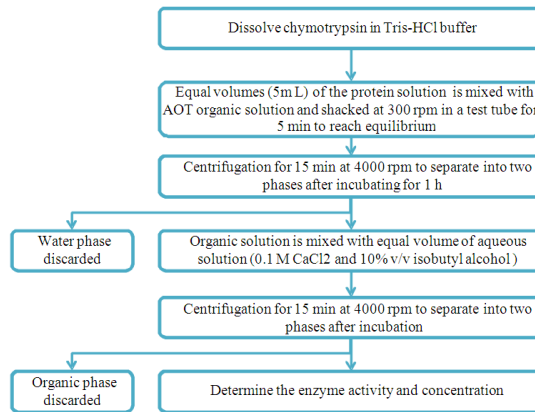

Fig. 6: The extraction of chymotrypsin from fish waste using reverse micelles method

The intestine is then chopped into small pieces and mixed with 50 mM Tris-HCl buffer having a pH of 7.5. The homogenized mixture is centrifuged at 10000 rpm and 4ºC for 15 minutes. Crude chymotrypsin (chymotrypsinogen) is extracted as a supernatant and stored at -80ºC till further purification (Heu *et al*., 1995; Chong *et al*., 2001; Li *et al*., 2005; Castillo-Yañez *et al*., 2009; Yang *et al*., 2009).

Fig. 7: The extraction of chymotrypsin from fish waste using chromatography

Purification: The chymotrypsinogen purification processes account for up to 80% of the total cost of chymotrypsin production. Precipitation is the technique most widely used in the chymotrypsin production industry because it can effectively purify and concentrate chymotrypsin at a very low cost (Matsudo *et al*., 2003; Boeris *et al*., 2009).Generally, chymotrypsin precipitation is used to obtain crude chymotrypsinogen and can be combined with chymotography techniques for further purification. Purification can be accomplished either by precipitation (using Tris-HCl) and ammonium sulfate fractionation or formation of reverse micelles (Mockel and Barnard, 1969; Simpson, 2000; Wilk, 2001;. Castillo- Yañez *et al*., 2009; Yang *et al*., 2009).

Ammonium sulfate method: Ammonium sulfate is the most reported polyelectrolyte in chymotrypsinogen production (Chatterjee *et al*., 2004). Folk (1970) used ammonium sulfate fractionation as second step inchymotrypsin purification process after extracted crude chymotrypsin with acetone powder. Same method had been used by Castillo-Yañez *et al*. (2006), Lam *et al*. (1999) and Möckel and Barnard (1969a). In the precipitation process, poly-charged molecules which contain opposite electrical charges to the chymotrypsinogen are added into protein solutions to form a chymotrypsinoge n-polyelectrolyte complex and generate insoluble aggregates. The materials used in the process are salts, non-ionicpolymers, organic solvents and polyelectrolytes which are easy to bioseparate in the downstream process (Boeris *et al*., 2009; Chatterjee*et al*., 2004). Yang (2009) used ammonium

sulfate saturation 30-60% in chymotrypsin extraction; Kristjansson and Nielson (1992), Castillo-Yañez *et al.* (2006, 2009) and Heu (1995) used ammomium sulfate saturation 30-70% and the recovery yield were in the rang from 55-60%. Boeris (2009) used Polyvinyl Sulfonate (PVS) as polyelectrolytes during the purification process to produce a chymotrypsinogen-PVS precipitate complex in acid pH which decreased the recovery rate of chymotrypsin. Also, increasing the PVS concentration lead to a lower recovery rate but produced a higher purity.

***Reverse micelles method*:** Reverse micelles are thermodynamically stable molecules that can extract large biomolecules like proteins through electrostatic interaction that attracts soluble proteins into the inner layer of the reverse micelles (Jolivalt *et al.*, 1990; Hu and Gulari, 1996). Reverse micelles are useful because they can form amphiphilic structures in polar organic media which can be used to extract large amounts of proteins in the aqueous phase without denaturation. In the purification process, selection of surfactants plays a significant role in protein stabilization. Sodium di-2-ethylhexyl sulfosuccinate (AOT) is the most common surfactant used in chymotrypsin purification. It can form reverse micelles without adding co-surfactant (Jolivalt *et al.*, 1990; Hu and Gulari, 1996; Hentsch *et al.*, 1992).

pH influences ionic molecular interactions in solution and, therefore, influences the efficiency of extraction by reverse micelles (Jolivalt *et al.*, 1990). The yield of chymotrypsin will increase with an increase in pH and the maximum extraction yield can be reached at an isoelectric point around a pH of 8.5 (Hu and Gulari, 1996). However, the pH of protein aqueous solutions should be held near a pH of 3 because at that pH the autohydrolysis rate is minimized and chymotrypsin is most stable (Hu and Gulari, 1996). Jolivalt *et al.* (1990) and Hentsch *et al.* (1992) reported that increasing chloride ion concentration will decrease chymotrypsin yield by competing with chymotrypsin in the extraction process and the effect is particularly significant at low ionic strength.

The whole extraction process with reverse micelles can be divided into two steps: forward-extraction and back-extraction. During the forward-extraction step, the aqueous and organic phases are separately prepared and homogenized with an orbital stirrer at 250 rpm for 90 min. After extraction (protein transfer from aqueous to organic phase) the phases are separated by centrifugation at 1500-2000 rpm for 10 min (Jolivalt *et al.*, 1990; Hu and Gulari, 1996). The concentration of protein can be measured with UV spectroscopy at 280 nm. Back-extraction transfers proteins from reverse micelles to aqueous solutions. Back-extraction is

usually very slow and $CaCl_2$ is added into the aqueous phase to assist the process (Hu and Gulari, 1996). The limitations of using reverse micelles in the back-extraction are due to: (a) the difficulty in separating proteins from the AOT reverse micellar phase and (b) the excessive time involved in the process (Hu and Gulari, 1996; Goto *et al.*, 1998).

Co-surfactants have been used to improve the extraction properties of AOT and the most common is Dioleyl Phosphoric Acid (DOPLA). This can be added to AOT to form AOT-DOLPA for use in chymotrypsin extract. Goto *et al.* (1998) reported that the effectiveness of mixed reverse micelles increased with increasing amount of DOLPA added. He also found that 4:1 ratio of AOT: DOLPA was the best for chymotrypsin extraction and that 10 mM of mixed reverse micelles had higher extraction ability than 200 mM of AOT. At low concentrations, the mixed reverse micells are very effective in separating and enriching chymotrypsin. Hu and Gulari (1996) and Goto *et al.* (1998) reported that mixed reverse micelles not only make chymotrypsin extraction more complete but it, also, shorten the back-extraction time from 24-2 h.

***Chromatography method*:** Chromatography is always the last step in the chymotrypsin purification process. Chromatography columns are used to concentrate and ultrafilter the fluid passing though them. The fluids can then be dialyzed against buffer (Folk, 1970). There are four types of chromatography that have been used in the chymotrypsin purification process: (a) ionic chromatography, (b) gel chromatography, (c) affinity chromatography and (d) hydrophobic interaction chromatography. Ionic chromatography is particularly useful because it can be used to both separate cationic and anionic forms of chymotrypsin A and B and to separate chymotrypsin from trypsin.

Heu *et al.* (1995) used gelfiltration chromatography with a Sephadex G-75 column (2.6×75 cm) to first isolate   chymotrypsin by molecular size. The crude chymotrypsin solution was further purified   using ion   chromatograph with a column   packed   with Diethylaminoethyl (DEAE)   cellulose, designed to interact with negatively charged proteins. Ryan (1965) used a similar column to purify chymotrypsin from chicken. Raae and Walther (1989) used both ion chromatograph with a DEAE-Sepharose column and gelfiltration with a Phenyl-Butyl-Amine-Sepharose (PBAS) column to purify chymotrypsin and eluted with 35-40% ethleneglycolin. Ionic chromatography with CM-cellulose was used in the double purification of chymotrypsin and chymotrypsinogen from turtle and fish equilibrated with 0.01M sodium succinate and

0.001M EDTA and eluted by salt gradients (Möckel and Bamard, 1969a; Möckel and Bamard, 1969b). Yang *et al*. (2009) used ionic chromatography with DEAE-Sephacel to purify chymotrypsin from crucian.

The two detected peaks were: the unretained portion was cationic chymotrypsin (B) and the returned portion was anionic chymotrypsin (A) which was eluted by NaCl at concentration of 0.2M. After gel-filtration on Sephacryl S-200, the two active portions were respectively subjected to hydrophobic interaction chromatography using Phenyl-Sepharose and SP-Sepharose for further purification. Most of the contaminated proteins were removed by washing with ammonium sulfate with concentrations ranging from 0 to 1M. The unretained ionic chymotrypsin B was further purified with a SP-Sepharose cationic exchange column. The results of purification are shown in Fig. 8.

The affinity chromatography has been used by Fujiwara *et al*. (1974), Branchini and Ziolkowski (1979), Nishikata (1983) and Ahn and Chung (1985). In affinity chromatography, a column containing cross-linked insoluble polymer or gel attached with a competitive chymotrypsin inhibitor or ligand is used (Ahn and Chung, 1985; Cuatrecasas, 1970). During the chromatography all other unwanted protein will pass through the column but chymotrypsin will be absorbed and bonded through extended hydrocarbon chains which make the ligand at varying distances from the gel matric backbone. The whole process is pH depended because the affecst the ionization of functional group. Chymotrypsin can be recovered by elution with 0.1 M acetic acid. Fujiwara *et al*. (1974) used carbobrnzoxyl-L-phenylalanyl-triethylenetetraminyl-Sepharose (Z-L-Phe-T-Sepharose), Cuatrecasas (1970) used Sepharose-bound D-tryptophan methyl ester Nishikata (1983) used sepharose with chymostatin analogue (Gly-Gly-L-Leu-L-phenylalaninal). Ahn and Chung (1985) used 4-phenylbutylamine as a ligand in chymotrypsin purification.

ACTIVATION OF CHYMOTRYPSINOGEN

Chymotrypsinogen is an inactive form of chymotrypsin existing in the pancreas and the extraction and purification processes result in pure chymotrypsinogen. Although chymotrypsinogen has enzymatic activity, the low level of activity makes it hard to detect in normal conditions. In order to evaluate the properties of chymotrypsin, chymotrypsinogen activation is a necessary process (Blow, 1976). Trypsin and other bacterial proteases excreted by Bacillus subtilis or Penicillium can be applied as a natural activator of chymotrypsin (Sakota, 1955; Dreyer and Neurrat, 1955; Prokuryakov, 1970). However, the optimal pHs of activators are different.
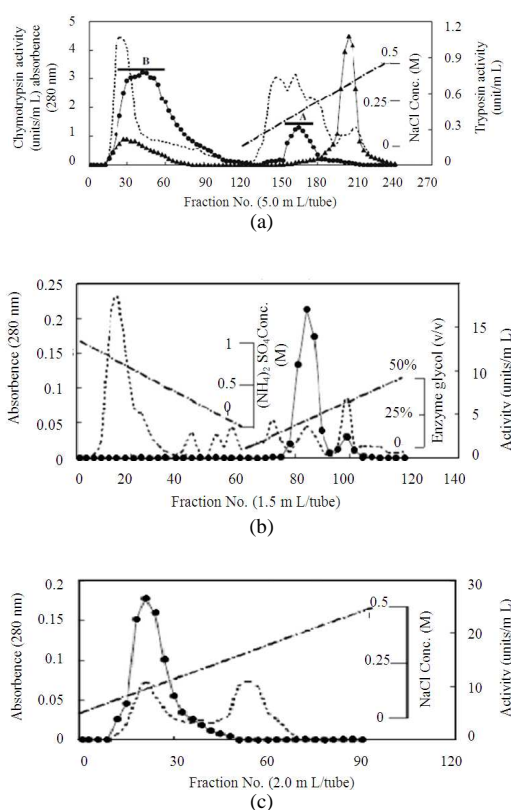

Fig. 8: Chromatographic purification of crucian carp chymotrypsins. (a) DEAE-Sepharose chromatography. (b) Phenyl-Sepharose chromatography. (c) SP-Sepharose hromatography. (●): Suc-Leu-Leu-Val-Tyr-AMC hydrolyzing activity. (▲): Boc-Phe-Ser-Arg MAC hydrolyzing activity (Yang *et al*., 2009)

The optimum pH for trypsin is 7.5, the optimal pH of Aspergillus oryzae is 3.0-5.0 and the optimal pH of Kaufman and Erlanger ranges from 3.2-3.4 (Prokuryakov, 1970). Since chymotrypsinogen is a single polypeptide chain made up of 245 amino acids, different forms of chymotrypsin can be activated by controlling the reaction condition (Boeris *et al*., 2009). In the whole process, trypsin only hydrolyzes one peptide bond between Arg 15 and Lie Ile 16 and forms π-chymotrypsin (Appel, 1986). The remainder of the N-terminal peptide plays an important role in protecting chymotrypsinogen from other non-specific activators by using disulfide bonds, which are connected to the left molecule (Spilliaert and Gudmundsdottir, 2000). Then, π-chymotrypsin autocatalyzes itself form chymotrypsin and three new disulphide bonds to link the polypeptide chain as shown in Fig. 9. A further

reaction will produce 2 types of chymotrypsin (δ-chymotrypsin and γ- chymotrypsin). Additionally, in the production of δ-chymotrypsin, it will lose the dipeptide Ser14-Arg15 bonds. Several factors contribute to chymotrypsinogen activation rates including concentration of trypsin, incubation time, temperature and pH.
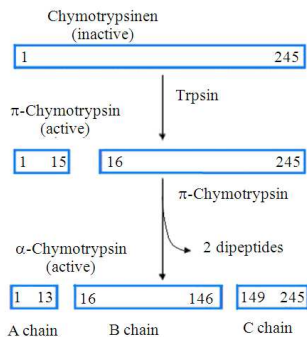

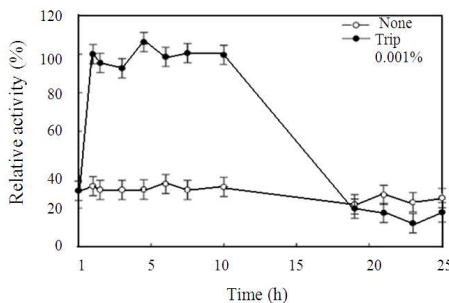Fig. 9: Activation of chymotrypsinogen. Peptide bonds split by trypsin and chymotrypsin in an autocatalytic process leading to defined chains and peptides (Berg *et al.*, 2007)

Fig. 10: ChTRP activity from bovine pancreas homogenate in a medium of sodium citrate-Tris-HCl 50 mM at a pH of 8.2 (Boeris *et al.*, 2009)
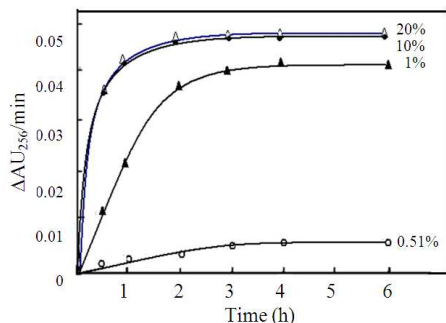

Fig. 11: Trypsin Requirement for Chymotrypsinogen Activation (Glazer and Steer, 1976)

Trypsin concentration: Trypsin is an activator of chymotrypsinogen which can dramatically accelerate the speed of zymogen activation even at a very low concentration (0.01 mg/g of homogenate). The relative activity of chymotrypsin during the zymogen activation process in the presence or absence of trypsin is shown in Fig. 10 (Berg *et al.*, 2007. Glazer and Steer (1976) found that an increase in trypsin concentrations at low concentrations (0.5-1.0%) did significantly increase the rate of activation but trypsin concentrations above 10% showed little increse in activation (Fig. 11). Thus, properly controlled concentration of trypsin in industry has commercial benefits.

Trypsin not only plays a significant role in chymotrypsinogen activation but also contributes to different types of final products. With a large presence of trypsin, π-chymotrypsin is rapidly formed and becomes the predominate product in the process and it further loses its dipeptide Ser 14-Arg 15 bonds. These bonds are autocatalytically produced by δ-chymotrypsin, which is known as rapid activation of chymotrypsinogen (Bettelheim and Neurath, 1954; Appel, 1986). Slow activation occurs in the presence of low concentrations of trypsin. The production of α-chymotrypsin is the major pathway and γ-chymotrypsin will be formed only slowly (Appel, 1986).

Incubation time: The incubation time required for chymotrypsinogen activation process depends on the activation method applied. There are two types of activation: classical activation (pH 7.5, 5°C, trypsin free, 48 incubation time) and rapid activation (activated by trypsin with incubation time varying from 1-48 h) (Bettelheim and Neurath, 1954; Miller *et al.*, 1971). With the addition of trypsin, the enzymogen activation significantly increases in 1 h with the decrease of thehomogenate solution viscosity. Sakota (1955) reported that the activity of chymotrypsin increases at a constant rate in the first 10 h followed by an observed decrease in catalytic activity after 24 h. The possible reason for decreased chymotrypsin activity could be that the non-active form of trypsin being self-generating in the solution and destroying the structure of chymotrypsinogen (Sakota, 1955; Boeris *et al.*, 2009). Engel and Alexander (1966) reported that activation of chymotrypsinogen was completed within 30 min.

Dreyer and Neurath (1955), Miller *et al.* (1971), Glazer and Steer (1976) reported that the activity of chymotrypsin was stable at 4°C and observed decreasing activity under high incubation temperature (Fig. 12).
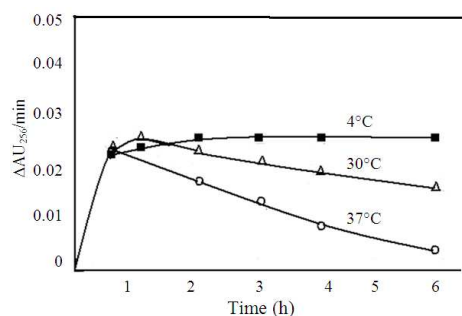
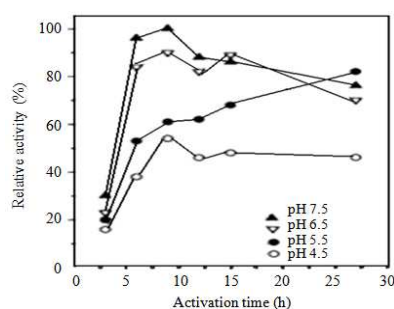Fig. 12: The temperature dependence of chymotrypsinogen (Glazer and Steer, 1976)

Fig. 13: pH effect on the activation of chymotrypsinogen (Guyonnet *et al*., 1999)

pH: Guyonnet *et al*. (1999) reported that the optimal pH for chymotrypsinogen activation is 7.5 (Fig. 13) and that the catalyze activity of chymotrypsin decreases with a decrease in pH. Similar results were reported by Engel and Alexander (1966), Bettelheim and Neurath (1954), Glazer and Steer (1976) and Guyonnet *et al*. (1999).

ASSAYING OF CHYMOTRYPSIN

Enzyme concentration: Three main methods are commonly used to evaluate chymotrypsin concentration: (a) absorbance at 280 nm (b) Bradford method and (c) Lowery method. Protein in solution has a maximum absorbance of ultraviolet light at 280nm. When measuring enzyme concentration, the wavelength must be adjusted to 280 nm and the system calibrated to zero with buffer solution. The absorbance of protein solution is then measured and the concentration (mg/mL) is calculated by the following equations (Layne, 1957; *et al*.; 2009; Stoscheck, 1990). This method had been used by Yang, Castillo-Yañez *et al*. (2006, 2009) and Möcke *et al*. (1969a).

For protein mixture:

$$\text{Concentration} = \frac{\Delta AU_{280}}{\text{path length (cm)}} \qquad (2)$$

For protein mixture with possible nucleic acid contamination:

$$\text{Concentration} = 1.55 \times \Delta AU280 - 0.76 \times \Delta AU_{260} \qquad (3)$$

In the Bradford method, Coomassie Blue reagent is mixed with the enzyme solution and the absorbance is read at 595 nm after incubation for 15 min. Concentrations are determined relative to standard curve based on Bovine Serum Albumin (BSA). The method had been used by Tsai (1986).

The method developed by Lowery *et al* (1951) is a relatively sensitive method but more complicated and time consuming compared to the Bradford Method. The enzyme solution is treated with Folin-ciocaltea reagents to create a blue compound and allowed to incubate for 10 min. Absorbance is read at 660 nm and standard using BSA is required. There are many substrates that have been reported to affect the results obtained from this method including Tris, EDTA, Sulfhydryl compounds, potassium compounds, disdulfide compounds, carbohydrates, glycerol, Tricine, detergents, most phenols, uric acid, guanine, magnesium and calcium (Olson and Markwell, 2007). This method had been widely used by Yang *et al*. (2009), Castillo-Yañez *et al*. (2006, 2009), Lam *et al*. (1999), Li *et al*. (2003).

Enzyme activity: A number of substrates are used to assay chymotrypsin activity including N-acetyl-L-Tyrosine Ethyl Ester (ATEE), Benzoyl-Tyrosine Ethyl Ester (BTEE) and N-Suc-Ala-Ala-Pro-Phe-*p*-Nitroanilide (SAAPPNA) (Hummel, 1959; Erlanger *et al*., 1961; Ramakrishna *et al*., 1987; Sabapathy and Teo, 1994; Heu *et al*., 1995; Chong *et al*., 2002; Chakrabarti *et al*., 2005; Li *et al*., 2005; Sveinsdóttir *et al*., 2006). The activity of chymotrypsin is determined as change in absorbance of chymotrypsin used in the assay per mg protein per min (Chakrabarti *et al*., 2005).

When ATEE is used as substrate, one unit of enzyme activity is defined as the decrease in measured absorbance of 0.0075 min^{-1} at 237 nm and 25°C. The enzyme is mixed with ATEE solution in potassiium phosphate buffer and the absorbance is measured every half min for 5 min. The activity is calculated using the following Eq. 4.

$$\text{Activity} = \frac{\Delta AU_{237}/\text{min} \times \text{dilution}}{0.0075 \times 0.2 \times \text{mg enzyme/ml original solution}} \qquad (4)$$

When BTEE is used as substrate, one unit of enzyme activity is defined as one unit of enzyme

hydrolyzing one micromole of BTEE per min at a pH of 7.5 and 25°C. The enzyme is added to BTEE solution dissolved in 50mM Tris-HCl buffer (10 mM $CaCl_2$ with a pH of 7.5) at room temperature and the absorbance is measured at 256 nm every half min for 5 min. The activity is calculated using the following Eq. 5 (Hummel, 1959; Sabapathy and Teo, 1994; Li *et al*., 2005; Parra *et al*., 2007; Tubio *et al*., 2009; Boeris *et al*., 2009).

$$Activity = \frac{\Delta AU_{256}/min \times 1000}{964 \times mg\ enzyme/ml\ in\ the\ reaction\ mixture} \quad (5)$$

When SAAPNA is used as a substrate, the enzyme activity is defined as one unit of enzyme activity hydrolyzing SAAPNA and releasing one micromole of p-nitroaniline at pH 7.5 and 25°C. The p-nitroaniline molar extinction coefficient is 8800 $M^{-1}cm^{-1}$. The enzyme is mixed with SAAPNA dissolve in 50 mM Tris-HCl buffer (10 mM $CaCl_2$ with a pH of 7.5) and absorbance is measured at 410 nm every half min for 5 min at room temperature. The activity is calculated using the following Eq. 6 (Hummel, 1959; Chong *et al*., 2002; Sveinsdóttir *et al*., 2006; Castillo-Yañez *et al*., 2006; Castillo-Yañez *et al*., 2009):

$$Activity = \frac{\Delta AU_{410}/min \times 1000 \times volume\ of\ reaction\ mixture}{8800 \times mg\ protein\ in\ the\ assay} \quad (6)$$

Molecular weight: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) has been widely used to determine molecular weight of chymotrypsin after extraction using zymograms as substrate (Garcia-Carreno *et al*., 1993; Heu *et al*., 1995; Chong *et al*., 2002). The SDS-PAGE consisted of 12% separating gel and 5% stacking gel. The extracted chymotrypsin is mixed with Tris-HCl buffer (pH 6.8) to make a proteinase sample (Chong *et al*., 2002). A five-microliter mixture of the chymotrypsin sample is loaded into the SDS-PAGE gel and the gel is dipped in a Tris-HCl buffer and 3% casein (pH 7.5) for 30 min at 5°C in order to allow the casein to enter the gel. After incubating the gel at 25°C for 60 min, the gel is washed, stained with Coomassie Brilliant Blue for 2 h and destained. Clear bands on the gel indicating enzyme activity are compared with molecule weight markers to indicate the molecule weight of chymotrypsin (Chong *et al*., 2002; Chakrabarti *et al*., 2006). Additionally, Sephacryl S-100 column (0.9×55 cm) gel filtration can also used to determine the molecule weight of chymotrypsin (Heu *et al*., 1995).

Effect of inhibitors: To study the effect of inhibitors on chymotrypsin activity, purified enzyme is incubated with several specific protease inhibitors such as Ethylenediaminetetraacetic Acid (EDTA), chymotrypsin specific inhibitors such as tosyl-phenylalanine Chloromethyl-Ketone (TPCK), serine protease inhibitors such as Soybean, Trypsin Inhibitor (SBTI) and Phenyl-Methyl-Sulphonyl-Fluoride (PMSF), benzamidine, 4-(2-Aminoethyl)-Benzenesulfonyl Fluoreide (AEBSF), leupeptin, benzamidine, ela-statinal or Tosyl-L-Lysine Chloromethyl Ketone (TLCK) (Lam *et al*., 1999; Castillo-Yañez *et al*., 2006; Castillo-Yañez *et al*., 2009; Yang *et al*., 2009). After incubation, substrate solutions are added and the residual activity is measured. The percentage activity was calculated using the activity of the blank as 100% (Lam *et al*., 1999; Castillo-Yañez *et al*., 2006; Castillo-Yañez *et al*., 2009; Yang *et al*., 2009).

Isoelectric point: The Isoelectric Point (IP) of chymotrypsin is always measured by analytical electrofocusing in thin-layer polyacrylamide flat get (LKB ampholyne PAG plate) containing ampholyne in the pH range of 3.5-9.5 (Castillo-Yañez *et al*., 2006; Castillo-Yañez *et al*., 2009). Purified protein is stained by Coomassie Brilliant Blue. The result is compared with isoelectric focusing calibration kit with 11 IP known proteins (Gildberg *et al*., 1990; Castillo-Yañez *et al*., 2006; Castillo-Yañez *et al*., 2009).

CONCLUSION

Chymotrypsin is an important digestive enzyme which widely exists in mammal pancreatic tissues and fish guts (intestine). It is a endopeptide compound with 245 amino acid and molecular weights ranging for 22.000-30,000 Da. Chymotrypsin specifically hydrolyzes peptide bounds with α-amino acid carbonyl groups, nonpolar aromatic group and nonpolar groups, it is inhibited by synthetic chymotrypsin inhibitors such as TPCK, ZGGPCK, ZPCK, chymostatin and PMSF as well as natural inhibitors from egg white, lima beans, potatoes and pigeonpea.

Three types of chymotrypsin (A, B and C) have been found in mammal pancreatic tissue but only two chymotrypsins (A and B) are found in fish. The optimal pH range for fish chymotrypsin is between 7.5-11 which is slightly higher than mammalian chymotrypsin. Fish chymotrypsin is more stable in basic environment than the acidic one. The optimal temperature for fish chymotrypsin is lower than mammal chymotrypsin. Most cold-water fish chymotrypsins have been reported to have 3-5 fold higher catalytic activity than mammalian chymotrypsin. The factors affecting

activity and the amount of chymotrypsin in fish are water temperature, fish species, fish age, fish weight, fish weight, starvation and nutrition.

Buffer extraction, ammonium sulphate precipitation and chromatography are usually used to produce chymotrypsin from fish. Each method has its own merit and demerit. Buffer extraction and ammonium sulphate precipitation can be used in large scale applications but the enzyme purity and activity is very low which can only be used in the leather or chemical industry. For the food and clinical industry, they require the use of higher purity and activity chymotrypsin, thus chromatography is needed. However, chromatography is very costly and it can only be applied on a small scale. Reverse micelles has been used for commercial chymotrypsin purification which can give a higher purity on a larger scale but no reports about purifying the chymotrypsin from raw materials have been found. Further study should focus on the optimization of purifying chymotrypsin from fish processing waste.

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