Songklanakarin J. Sci. Technol.
30 (1), 37-46, Jan - Feb 2008

Songklanakarin Journal
of Science and Technology

http://www.sjst.psu.ac.th

Review Article

Digestive proteinases from marine organisms and their applications

Sappasith Klomklao*

Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phattalung Campus, Phattalung, 93110, Thailand.

Received 20 March 2007; Accepted 26 February 2008

Abstract

Fish viscera have wide biotechnological potential as a source of digestive enzymes, especially proteinases. The biological diversity of fish species provides a wide array of enzymes with unique properties. Fish digestive proteolytic enzymes most commonly found include pepsin and trypsin. Those enzymes from fish viscera may have the advantages for the applications in the food industry since their temperature and other characteristics differ from homologous proteinases from warm-blooded animals. Therefore, digestive proteinases can be isolated as a value-added product from fish viscera and used as the processing aids in food industries to maximize the utilization of marine resources.

Keywords: viscera, digestive proteinase, application, trypsin, pepsin, hydrolysis

1. Introduction

Large quantities of fish processing wastes are produced worldwide annually. Viscera, head, bones and frames, stick-water and effluent from processing account for more than 70% of the total weight of some aquatic animals. Javeed and Mahendrakar (1996) reported that fish internal organs constitute approximately 7.5% of body weight. The discards from fish processing together with fish by-products pose great disposal problems for management. Stom and Eggum (1981) reported that fish internal organs and heads were converted to powdered fish flour used as animal feed. Those wastes can also be used for fish protein hydrolysates (FPH) production (Benjakul and Morrissey, 1997; Shahidi *et al.*, 1995). The hydrolysates have a wide range of potential applications, e.g. as ingredients in animal feed (Faid *et al.*, 1997) or food (Frokjaer, 1994; Lahl and Braun, 1994), as the peptone for microbial growth media (Vecht-Lifshitz *et al.*, 1990, or as fertilizer (Kurbanoglu and Algur, 2002). Among by-products, fish viscera are potential sources of enzymes. The recovery of proteinases from fishery by-products is of great importance since low-cost proteinases could promote new industrial application. (Simpson, 2000; Klomklao et al., 2004). In recent years, recovery and characterization of enzymes from marine fish have been carried out and these have led to the emergence of some interesting new applications of these enzymes in food processing. Extraction of enzymes from fish processing wastes and their utilization in the food industry may contribute significantly to reducing local pollution problem (Haard, 1992). The purpose of this review is to provide an overview of proteinases from marine digestive organisms and to discuss their potential uses in the food industry.

2. Digestive proteinases from marine fish

Proteinases play an essential role in the growth and survival of all living organisms. The hydrolysis of peptide bonds catalyzed by proteinases is a common reaction in nature. For marine animals, proteinases are mainly produced by the digestive glands. Like the proteinases from plants, animals and microorganisms, digestive proteinases from marine animals are polyfunctional enzymes catalyzing the hydrolytic degradation of proteins (Garcia-Carreno and Hernandez-Cortes, 2000). Marine animals have adapted to different environmental conditions, and these adaptations,

*Corresponding author.
Email address: sappasith@tsu.ac.th

together with inter- and intraspecies genetic variations, are associated with certain unique properties of their proteinases, compared with their counterpart enzymes from land animals, plants and microorganisms (Simpson, 2000). Some of these distinctive properties include higher catalytic efficiency at low temperature and lower thermal stability (Klomklao *et al.*, 2005).

Digestive proteinases have been studied in s everal species of fish (De Vecchi and Coppes, 1996) and decapods (Garcia-Carreno and Haard, 1993). Proteinases found in the digestive organs of fish include pepsin, gastricsin, trypsin, chymotrypsin, collagenase, elastase, carboxypeptidase and carboxyl esterase (Haard, 1994; Simpson, 2000). Pepsin and trypsin are two main groups of proteinases found in fish viscera. Pepsin is localized in fish stomach (Gildberg *et al.*, 1990), while trypsin is concentrated in pyloric ceca and intestine (Kishimura *et al.*, 2006a). The distribution of proteinase varies, depending on species and organs. Torrissen (1984) reported that proteinase activity from intestine of rainbow trout (*Salmo gairdneri*) was higher than that of Atlantic salmon (*Salmo salar*). Pyloric ceca of chinook salmon (*Oncorhynchus tschawytscha*) had a higher proteinase activity than that of rainbow trout (Dimes *et al.*, 1994). For discus fish (*Symphysodon aequifasciata*), proteinase activity in intestine was higher than that in stomach (Chong *et al.*, 2002). The varying distribution of proteinase activity in individual internal organ of three tuna species including skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacores*) and tongol tuna (*Thunnus tonggol*) commercially used in Thai tuna industries was reported by Klomklao *et al.* (2004).

2.1 Classification of digestive proteinases from marine animals

Digestive proteinases from marine animals may be classified by the same criteria used for proteinases from other animals, plants or microorganisms, on the basis of their similarity to well characterized proteinases as trypsin-like, chymotrypsin-like, chymosin-like or cathepsin-like. They may be classified on the basis of their pH sensitivities as acid, neutral, or alkaline proteinases. They are also characterized by common names and trade names, preferential specificity and response to inhibitor specificity. In EC system for enzyme nomenclature, all proteases (peptide hydrolases) belong to subclass 3.4, which is further divided into 3.4.11-19, the exopeptidases and 3.4.21-24, the endopeptidases or proteinases (Nissen, 1993). Endopeptidases cleave the polypeptide chain at particularly susceptible peptide bonds distributed along the chain, whereas exopeptidases hydrolyze one amino acid from N terminus (amino peptidases) or from C terminus (carboxypeptidases) (Figure 1). Exopeptidases, especially aminopeptidases, are ubiquitous, but less readily available as commercial products, since many of them are intracellular or membrane bound.

Based on the nature of catalytic site, digestive proteinases from marine animals are further classified into four categories as acid or aspartate proteinases, serine proteinases, thiol or cysteine proteinases, or metalloproteinases (Simpson, 2000). The enzymes in the different classes are differentiated by various criteria, such as the nature of the groups in their catalytic sites, their substrate specificity, their response to inhibitors or by their activity/stability under acid or alkaline conditions (Nissen, 1993).

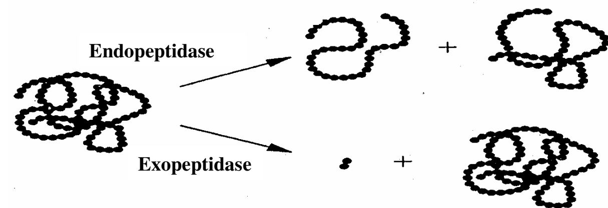

Figure 1. Action of endopeptidases and exopeptidases on protein structure (An *et al.*, 1996).

1) Acid/Aspartyl proteinases

The acid or aspartyl proteinases are a group of endopeptidases characterized by high activity and stability at acidic pH. They are referred to as “aspartyl” proteinases (or carboxyl proteinases) because their catalytic sites are composed of the carboxyl group of two aspartic acid residues (Whitaker, 1994). Based on the EC system, all the acid/aspartyl proteinases from marine animals have the first three digits in common as EC 3.4.23. Three common aspartyl proteinases that have been isolated and characterized from the stomach of marine animals are pepsin, chymosin, and gastricsin (Simpson, 2000).

Pepsin, one of the major proteinases found in fish viscera, has an extracellular function as the major gastric proteinase. Pepsin, secreted as a zymogen (pepsinogen), is activated by the acid in stomach to an active form (Clarks *et al.*, 1985). Pepsin is composed of a single polypeptide chain of 321 amino acids and has a molecular weight of 35 kDa (Simpson, 2000). However, the pepsins from marine animals were reported to have molecular weights ranging from 27 to 42 kDa. The molecular weights of two pepsins (I and II) from orange roughy stomach were estimated to be approximately 33.5 and 34.5 kDa, respectively (Xu *et al.*, 1996). Sanchez-Chiang *et al.* (1987) reported that the molecular weights of two pepsins from stomach of salmon were estimated to be approximately 32 and 27 kDa by gel filtration. Molecular weights of two pepsins from polar cod stomach were estimated by SDS-PAGE to be approximately 42 and 40 kDa (Arunchalam and Haard, 1985). Klomklao *et al.* (2007a) reported that pepsin A and B from stomach of pectoral rattail (*Coryphaenoides pectoralis*) had the apparent molecular weights of 35 and 31 kDa, respectively, as estimated by SDS-PAGE (Figure 2) and gel filtration on Sephacryl S-200.

Pepsins and pepsin-like enzymes can be extracted from the digestive glands of marine animals such as Atlantic cod (*Gadus morhua*) (Brewer *et al.*, 1984), capelin (*Mallotus*

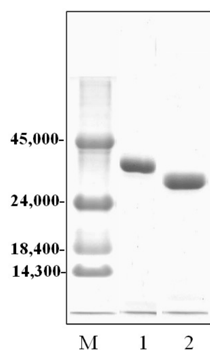

Figure 2. Protein pattern of purified pepsins A and B from pectoral rattail stomach determined by SDS-PAGE. M, molecular weight standard; lane 1, pepsin A; lane 2, pepsin B (Klomklao *et al.*, 2007a).

villosus) (Gildberg and Raa, 1983), polar cod (*Boreogadus saida*) (Arunchalam and Haard, 1985) sardine (*Sardinos melanostica*) (Noda and Murakami, 1981) and Montery sardine (*Sardinops sagax caerulea*) (Castillo-Yanez *et al.*, 2004). Several methods have been described in the literature for purification of pepsins from marine animals. Gildberg and Raa (1983) purified pepsins from stomach of Arctic capelin (*Mallotus villosus*) by ammonium sulfate precipitation, DEAE-cellulose and Sephadex G-75, respectively. Pepsin from polar cod stomach was isolated by CBZ-D-phenylalanine-TETA- Sepharose 4B (Arunchalam and Haard, 1985). Furthermore, Gildberg *et al.* (1990) purified pepsin from stomach of Atlantic cod (*Gadus morha*) by ammonium sulfate fractionation (20-70% saturation), followed by ion-exchange chromatography using S-Sepharose column. Recently, Klomklao *et al.* (2007a) purified two pepsins, A and B, from the stomach of pectoral rattail by acidification, ammonium sulfate precipitation (30-70% saturation), followed by a series of column chromatographies including Sephacryl S-200, DEAE-cellulose and Sephadex G-50.

Pepsin activity is very dependent on pH values, temperatures and type of substrate. Hemoglobin is the substrate most frequently used for determination of pepsin activity (De Vecchi and Coppes, 1996; Klomklao *et al.*, 2004). Haard (1986) reported that the initial rate of hemoglobin digestion by Atlantic cod pepsin was maximal at 35°C and pH 1.9. Pepsin from polar cod stomach exhibited a maximal activity against hemoglobin at pH 2.0 and 37°C (Arunchalam and Haard, 1985). Gildberg *et al.* (1990) reported that the optimal pH of Atlantic cod pepsin for hemoglobin hydrolysis was 3.0. Fish pepsins were shown to hydrolyze hemoglobin much faster than casein (Gildberg and Raa, 1983). Most fish species contain two or three major pepsins with an optimum hemoglobin digestion at pH between 2 and 4 (Gildberg and Raa, 1983). Gildberg *et al.* (1990) found that the affinity of cod pepsin, especially pepsin I towards hemoglobin, was lower at pH 2 than at pH 3.5. Klomklao *et al.* (2007a) also found that pepsin A and B had maximal activity at pH 3.0 and 3.5, respectively, and had the same optimal temperature at 45°C using hemoglobin as a substrate (Figure 3).

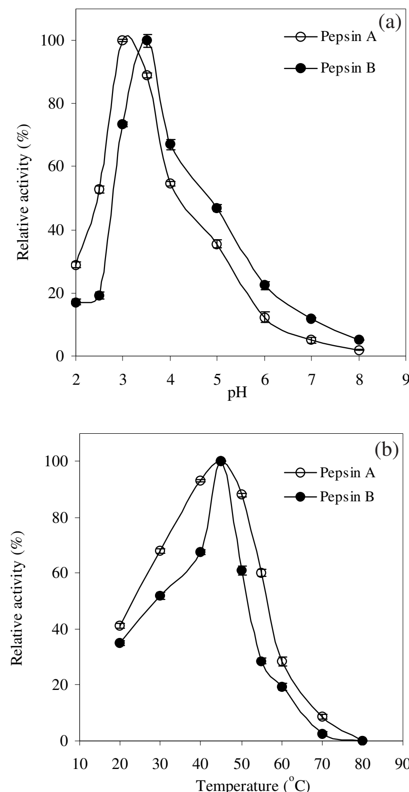

Figure 3. pH (a) and temperature (b) profiles of purified pepsins A and B from pectoral rattail stomach (Klomklao *et al.*, 2007a).

For pH stability, pepsin is quite stable from pH 2 to about 6 but it rapidly loses activity at pH above 6 due to the denaturation (Simpson, 2000). Pepsin from sardine stomach was stable between pH 2 and 6 and showed drastic loss of activity at pH 7.0 (Noda and Murakami, 1981). Castillo-Yanez *et al.* (2004) found that Monterey sardine acidic enzymes were stable at pH ranging from 3.0 to 6.0. Klomklao *et al.* (2007a) also reported that both pepsins A and B from the stomach of pectoral rattail were stable in the pH range of 2.0-6.0 (Figure 4).

Chymosin have been described as acid proteinases with some characteristics distinct from other acid proteinases. For example, these enzymes are most active and stable around pH 7.0 unlike other acid proteinases. They also have relatively narrower substrate specificity, compared with other acid proteinases such as pepsin (Simpson, 2000). Digestive proteinases with chymosin activity were isolated as zymogens from the gastric mucosa of young and adults seals (*Pagophilus groenlandicus*) (Shamsuzzaman and Haard, 1984) by a series of chromatographies including DEAE-Sephadex A-50, Sephadex G-100 and Z-D-Phe-T-Sepharose gel. The enzyme had optimal pH of 2.2-3.5 for hemoglobin hydrolysis. The chymosins from marine animals did not hydrolyze the specific synthetic substrate for pepsin (i.e., *N*-acetyl-L-phenylalanine diiodotyrosine (APD)) and were also

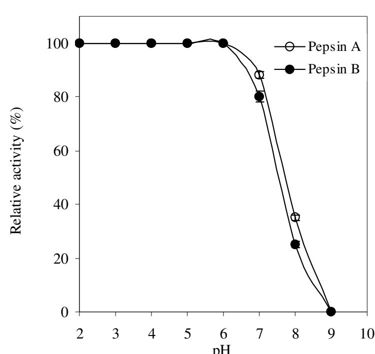

Figure 4. pH stability of purified pepsins A and B from pectoral rattail stomach (Klomklao *et al.*, 2007a).

more susceptible to inactivation by urea (Simpson, 2000).

Gastricsins, like pepsins, are aspartyl proteinases that have many properties in common with other enzymes in the family of gastric proteinases. However, they differ from pepsins in structure and certain catalytic properties (Simpson, 2000). Two gastricsin isozymes were purified and characterized from the gastric juices of hake (Sanchez-Chiang and Ponce, 1981). The optimum pH for the hydrolysis of hemoglobin by hake gastricsins was 3.0, which was similar to that of mammalian gastricsins. Hake gastricsins were stable up to pH 10 but rapidly inactivated at higher pH values (Sanchez-Chiang and Ponce, 1981). The latter property would appear to distinguish the gastricsins from pepsins (Simpson, 2000).

2) Serine proteinases

Serine proteinases have been described as a group of endoproteinases with a serine residue in their catalytic site. This family of proteinases is characterized by the presence of a serine residue, together with an imidazole group and aspartyl carboxyl group in their catalytic sites (Simpson, 2000). The proteinases in serine subclass all have the same first three digits: EC 3.1.21. Trypsin and chymotrypsin are the major serine proteinases purified and well characterized from the digestive glands of marine animals.

Trypsins (EC 3.4.21.4), mainly members of a large family of serine proteinases, specifically hydrolyze proteins and peptides at the carboxyl side of arginine and lysine residues (Klomklao *et al.*, 2006a). Trypsins play major roles in biological processes including digestion, activation of zymogens of chymotrypsin and other enzymes (Cao *et al.*, 2000). Trypsins from marine animals resemble mammalian trypsins with respect to their molecular size (22-30 kDa), amino acid composition and sensitivity to inhibitors. Their pH optima for the hydrolysis of various substrates were from 7.5 to 10.0, while their temperature optima for hydrolysis of those substrates ranged from 35 to 65°C (De Vecchi and Coppes, 1996).

A number of studies on trypsins from fish viscera have been carried out. Martinez *et al.* (1988) purified two trypsin-like enzymes (trypsin A and trypsin B) from the pyloric ceca and intestine of anchovy (*Engraulis encrasicholus*) by ammonium sulfate fractionation, affinity chromatography (benzamidine-Sepharose 6B) and ion-exchange chromatography (DEAE-Sepharose), respectively. Molecular weight of trypsins A and B were estimated to be 27 kDa and 28 kDa, respectively. Optimum pHs for both proteinases were 8-9.

Two anionic trypsins (trypsin A and trypsin B) from the hepatopancreases of carp were purified using a series of chromatographies including DEAE-Sephacel, Ultrogel AcA54 and Q-Sepharose (Cao *et al.*, 2000). Trypsin A was purified to homogeneity with a molecular weight of 28 kDa, while trypsin B showed two close bands of 28.5 kDa and 28 kDa on SDS-PAGE. Trypsin A and B had optimal activity at 40 and 45°C, respectively, and had the optimum pH of 9.0 using Boc-Phe-Ser-Arg-MCA as a substrate. Both enzymes were effectively inhibited by trypsin inhibitors. Trypsin from the pyloric ceca of Monterey sardine (*Sardinops sagax caerulea*) with molecular weight of 25 kDa was purified and characterized by Castillo-Yanez *et al.* (2005). The optimum pH for activity was 8.0 and maximal activity was found at 50°C. The purified enzyme was partially inhibited by 1.4 mg/ml PMSF and fully inhibited by 0.5 mg/ml soybean trypsin inhibitor and 2.0 mg/ml benzamidine, but was not inhibited by the metalloproteinase inhibitor, 0.25 mg/ml EDTA.

Additionally, two trypsins, TR-S and TR-P, were purified from the viscera of true sardine (*Sardinops melanostictus*) and pyloric ceca of arabesque greenling (*Pleuroprammus azonus*) by gel filtration using Sephacryl S-200 and Sephadex G-50 and anion-exchange chromatography using DEAE-cellulose (Kishimura *et al.*, 2006b). The TR-S and TR-P had maximal activities at around pH 8.0 for hydrolysis of TAME. Optimum temperatures of TR-S and TR-P were 60°C and 50°C, respectively. Trypsin was reported to be the major form of proteinase in the spleen of tongol tuna (*Thunnus tongol*) based on the molecular weight, the inhibition by TLCK and the activity toward specific substrates (Klomklao *et al.*, 2006a). Two anionic trypsins (A and B) were purified from yellowfin tuna (*Thunnus albacores*) spleen. Trypsins A and B exhibited the maximal activity at 55 and 65°C, respectively, and had the same optimal pH at 8.5 using TAME as a substrate (Klomklao *et al.*, 2006b). Recently, Klomklao *et al.* (2007b) purified trypsins from skipjack tuna (*Katsuwonus pelamis*) spleen by a series of chromatographies including Sephacryl S-200, Sephadex G-50 and diethylaminoethyl-cellulose. Skipjack tuna spleen contained three trypsin isoforms, trypsins A, B and C. The molecular weight of all trypsin isoforms was estimated to be 24 kDa by size exclusion chromatography on Sephacryl S-200 and SDS-PAGE. The optimum pH and temperature for TAME hydrolysis of all trypsin isoforms were 8.5 and 60°C, respectively (Figure 5).

Trypsins from marine animals tend to be more stable at alkaline pH, but are unstable at acidic pH. On the other hand, mammalian trypsins are most stable at acidic pH (Simpson, 2000; Klomklao *et al.*, 2006a). Trypsin from

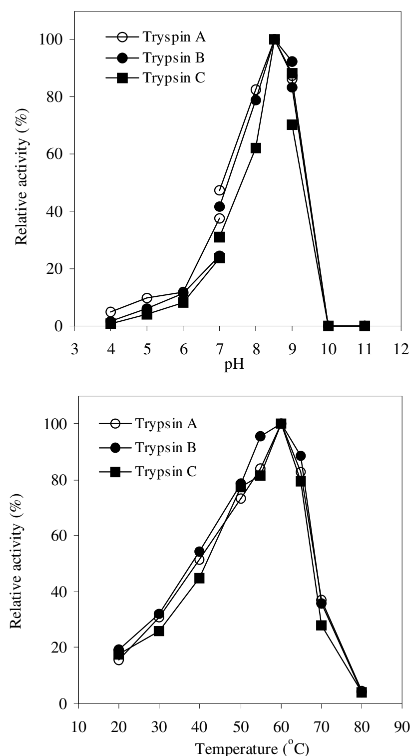

Figure 5. pH (a) and temperature (b) profiles of purified trypsins A, B and C from skipjack tuna spleen (Klomklao *et al.*, 2007b).

tongol tuna spleen showed the high stability in the pH range of 6-11, but the inactivation was more pronounced at pH values below 6 (Klomklao *et al.*, 2006a). Klomklao *et al.* (2007b) also reported that skipjack tuna spleen trypsins were stable in the pH ranging from 6.0 to 11.0 but were unstable at pH below 5.0 (Figure 6). The stability of trypsins at a particular pH might be related to the net charge of the enzyme at that pH (Castillo-Yanez *et al.*, 2005). Trypsin might undergo the denaturation under acidic conditions, where the conformational change took place and enzyme could not bind to the substrate properly (Klomklao *et al.*, 2006a).

N-terminal amino acid sequences are useful as tools to identify the type of enzymes and may be useful for design-

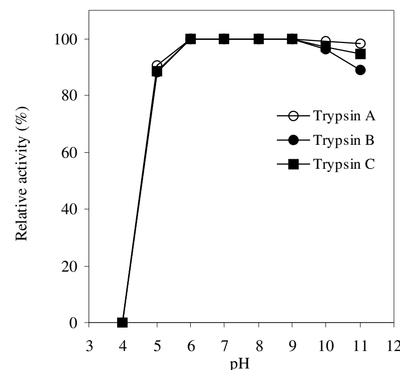

Figure 6. pH stability of purified trypsins A, B and C from skipjack tuna spleen (Klomklao *et al.*, 2007b).

ing primers for cDNA cloning of enzyme (Cao *et al.*, 2000; Klomklao *et al.*, 2007b). Table 1 showed the N-terminal amino acid sequences of fish trypsins compared with those of mammals. Generally, fish trypsins had a charged Glu residue at position 6, where Thr is most common in mammalian pancreatic trypsins (Table 1). Moreover, the sequence of all trypsins started with IVGG after limited proteolysis of inactive trypsinogen into the active trypsin form (Cao *et al.*, 2000; Klomklao *et al.*, 2006b).

Chymotrypsin is another member of a large family of serine proteinases functioning as a digestive enzyme. Chymotrypsins have been isolated and characterized from marine species such as anchovy (Heu *et al.*, 1995), Atlantic cod (Asgiersson and Bjarnason, 1991) and Monterey sardine (Castillo-Yanez *et al.*, 2006). In general, these enzymes are single-polypeptide molecules with molecular weights between 25 and 28 kDa. They are most active within the pH range of 7.5 to 8.5 and are most stable at around pH 9.0 (Simpson, 2000). Chymotrypsin has a much broader specificity than trypsin. It cleaves peptide bonds involving amino acids with bulky side chains and nonpolar amino acids such as tyrosine, phenylalanine, tryptophan, and leucine (Simpson, 2000).

3) Thiol/Cysteine proteinases

Thiol or cysteine proteinases are a group of endo-

Table 1. N-terminal amino acid sequence of fish and mammalian trypsins.

Source of trypsin	N-terminal sequence	Reference
Skipjack tuna	IVGGYECQAHSQPHQVSLNS	Klomklao *et al.* (2007b)
Yellowfin tuna	IVGGYECQAHSQPHQVSLNA	Klomklao *et al.* (2006a)
Tongol tuna	IVGGYECQAHSQPHQVSLNA	Klomklao *et al.* (2006b)
True sardine	IVGGYECKAYSQPWQVSLNS	Kishimura *et al.* (2006b)
Japanese anchovy	IVGGYECQAHSQPHTVSLNS	Kishimura *et al.* (2005)
Cod	IVGGYECTKHSQAHQVSLNS	Gudmundsdottir *et al.* (1993)
Salmon	IVGGYECKAYSQTHQVSLNS	Male *et al.* (1995)
Porcine	IVGGYTCAANSVPYQVSLNS	Hermodson *et al.* (1973)
Bovine	IVGGYTCGANTVPYQVSLNS	Walsh (1970)

peptidases that have cysteine and histidine residues as the essential groups in their catalytic sites. These enzymes require the thiol (-SH) group furnished by the active site cysteine residue to be intact, hence this group is named "thiol" or "cysteine" proteinases (Mihalyi, 1978). The thiol proteinases are inhibited by heavy metal ions and their derivatives, as well as by alkylating agents and oxidizing agents (Mihalyi, 1978). The first three digits common to thiol proteinases are EC 3.4.22.

Digestive cysteine or thiol proteinases have been found in digestive organ of marine animals. Marine digestive cysteine proteinases are most active at acidic pH and inactive at alkaline pH. Common examples of digestive thiol proteinase from marine animals are cathepsin B, cathepsin L and cathepsin S (Simpson, 2000). Various researchers have described different procedures for isolating marine cysteine or thiol proteinases from the digestive glands of marine animals.

Cathepsin B was isolated from a few aquatic animals including the horse clam (Reid and Rauchert, 1976) and mussel (Zeef and Dennison, 1988). Generally, cathepsin B from marine animals is single polypeptide chain with molecular sizes ranging from 13.6 to 25 kDa. Cathepsins from different species display maximum activity over a broad pH range of 3.5-8.0. Cathepsin B is activated by Cl^- ions, and requires sulfhydryl-reducing agents or metal-chelating agents for activity (Zeef and Dennison, 1988).

Cathepsin L from carp hepatopancreas was purified by using ammonium sulfate precipitation and a series of chromatographies, in which the enzyme had an affinity toward Concanavalin A and Cibacron Blue F3GA (Aranishi *et al.*, 1997). Its homogeneity was established by a native-PAGE. Two protein bands corresponding to molecular weights of 30 kDa and 24 kDa were found on SDS-PAGE. The enzyme exhibited a maximal activity against Z-Phe-Arg-MCA at pH 5.5-6.0 and 50°C. All tested cysteine proteinase inhibitors, TLCK and chymostatin, markedly inhibited its activity, whereas the other serine proteinase inhibitors and metalloproteinase inhibitor showed no inhibitory activity on the enzyme (Aranishi *et al.*, 1997).

Cathepsin S from hepatopancreas of carp (*Cyprinus carpio*) was purified by ammonium sulfate fractionation, followed by SP-Sepharose, Sephacry S-200 and Q-Sepharose, respectively (Pangkey *et al.*, 2000). The molecular weight of purified proteinase was 37 kDa estimated by SDS-PAGE. It hydrolyzed Z-Phe-Arg-MCA but not Z-Arg-MCA. The optimal pH and temperature for the hydrolysis of Z-Phe-Arg-MCA were 7.0 and 37°C, respectively. This proteinase activity was inhibited by E-64, leupeptin, 5-5'-dithiobis (2-nitro-benzoic acid) and *p*-tosyl-lys-chloromethylketone.

4) Metalloproteinases

The metalloproteinases are hydrolytic enzymes whose activity depends on the presence of bound divalent cations (Simpson, 2000). Chemical modification studies suggest that there may be at least one tyrosyl residue and one imidazole residue associated with the catalytic sites of metalloproteinases (Whitaker, 1994). The metalloproteinases are inhibited by chelating agents such as 1, 10-phenanthroline, EDTA, and sometimes by the simple process of dialysis. The metalloproteinases have been characterized from marine animals (e.g., rockfish, carp, and squid mantle) but have not been found in the digestive glands except in the muscle tissue (Simpson, 2000). Metalloprotienases do not seem to be common in marine animals (Simpson, 2000). However, Sivakumar *et al.* (1999) purified collagenolytic metalloproteinase with gelatinase activity from carp hepatopancreas by ammonium sulfate fractionation and gel filtration chromatography. The enzyme had a molecular weight of 55 kDa and was active against native type I collagen. Optimum temperature and pH were 25°C and 7-7.5, respectively. Activity of active enzyme was strongly inactivated by 10 mM EDTA.

2.2 Applications of fish digestive proteinases

Proteinases are by far the most studied enzymes for industrial bioprocessing. Almost half of all industrial enzymes are proteinases, mostly used in the detergent, leather and

Table 2. Some uses of proteolytic enzymes in food industry. (Haard, 1992)

Commodity	Application
Cereals, baked goods	Increase drying rate of proteins; improve product handling. Characteristics; decrease dough mixing time; improve texture and loaf volume of bread; and decrease dough mixing time
Egg and egg products	Improve quality of dried products
Meats	Tenderization; recover protein from bones; hydrolysis of blood proteins
Fish	Fish protein hydrolysates, viscosity reduction, skin removal, roe processing
Pulses	Tofu; soy sauce; protein hydrolysis; off-flavor removal soy milk
Dairy	Cheese curd formation; accelerate cheese aging; rennet puddings
Brewing	Fermentation and filtration aid; chill proofing Clarification; decrease foaming, promote malolactic fermentation
WineCoco	Facilitate fermentation for chocolate production

food industries (Klomklao *et al.*, 2005). The food industry uses proteases as processing aids for many products including baked goods, beer, wine, cereals, milk, fish products, and legumes. Production of protein hydrolysates and flavor extracts has been known to be successful by using selected proteases (Table 2) (Stefansson, 1988; Wray, 1988; Haard, 1990; Wasserman, 1990; Simpson and Haard, 1987).

Commercial supplies of food processing enzymes are presently derived from various plants, animal and microbial sources. Compared with these enzymes used in food processing, the use of proteinases from marine fish is still rare. Fish digestive enzymes can be recovered as by-products from fish processing wastes (Stefansson and Steingrimsdottir, 1990; Haard, 1992). However, most proteinases from marine organisms are extracellular digestive enzymes with characteristics differing from homologous proteases from warm-blooded animals (De Vecchi and Coppes, 1996). They are more active catalysts at relatively low temperature, compared with similar enzymes from mammals, thermophylic organisms and plants (Simpson and Haard, 1987). Low temperature processing could provide various benefits, such as low thermal costs, protection of substrates or products from thermal degradation and/or denaturation, and minimization of unwanted side reactions (Haard, 1992). Certain fish enzymes are excellent catalysts at low temperature, which is advantageous in some food processing operations (Simpson and Haard, 1987; Simpson, 2000). For example, cold-adopted pepsins are very effective for cold renneting milk clotting because of the high activity at low reaction temperatures (Brewer *et al.*, 1984). Moreover, fish digestive enzymes possessing other unique properties might make them better suited as food processing aids.

Recently, the use of alkaline proteinases from marine digestive organs, especially trypsin, has increased remarkably, since they are both stable and active under harsh conditions, such as at temperatures of 50 to 60°C, high pHs and in the presence of surfactants or oxidizing agents (Klomklao *et al.*, 2005). Trypsin can be used for extraction of carotenoprotein from shrimp processing wastes. Cano-Lopez *et al.* (1987) reported that using Atlantic cod trypsin from pyloric ceca in conjunction with a chelating agent (EDTA) in the extraction medium increased the efficacy in recovering both protein and pigment from crustacean wastes. This method has facilitated the recovery of as much as 80% of astaxanthin and protein from shrimp wastes as carotenoprotein complex. Recently, Klomklao *et al.* (2007c) also recovered carotenoproteins from black tiger shrimp waste by using trypsin from bluefish pyloric ceca. The product obtained contained higher protein and pigment content than those of black tiger shrimp waste and had low contents of chitin and ash (Table 3).

The enzymes recovered from fish have also been successfully used as seafood processing aids including the acceleration of fish sauce fermentation. Chaveesuk *et al.* (1993) reported that the supplementation with trypsin and chymotrypsin significantly increased protein hydrolysis of fish sauce. Fish sauce prepared from herring with enzyme supplementation contained significantly more total nitrogen, soluble protein, free amino acid content and total amino acid content, compared to fish sauce with no added enzyme (Chaveesuk *et al.*, 1993). By supplementing minced capelin with 5-10% enzyme-rich cod pyloric ceca, a good recovery of fish sauce protein (60%) was obtained after 6 months of storage (Gildberg, 2001). Additionally, Klomklao *et al.* (2006c) reported that fish sauce prepared from sardine with spleen supplementation contained greater total nitrogen (Figure 7), amino nitrogen, formaldehyde nitrogen, ammonia nitrogen content, than did those without spleen addition throughout the fermentation. Therefore, the addition of spleen can accelerate the liquefaction of sardine for fish sauce production.

Proteolytic enzymes also show the profound effects on collagen extraction. Generally, use of pepsin in combination with acid extraction increased in the yield of collagen. Nagai *et al.* (2002) reported that the yield of pepsin-solubilized collagen was higher (44.7%) than acid-solubilized collagen (10.7%). Nagai and Suzuki (2002) found that the collagen extracted from the outer skin of the paper nautilus was hardly solubilized in 0.5 M acetic acid. The insoluble matter was easily digested by 10% pepsin (w/v), and a large amount of collagen was obtained with 50% yield (pepsin-solubilized collagen). Collagen from the outer skin of cuttlefish (*Sepia lycidas*) was also extracted by Nagai *et al.* (2001). The initial extraction of the cuttlefish outer skin in acetic acid yielded only 2% of collagen (dry weight basis). With a subsequent digestion of the residue with 10% pepsin (w/v), a solubilized collagen was obtained with a yield of 35% (dry weight basis). Pepsin solubilized collagen was extracted from the skin of grass carp (*Ctenopharyngodon idella*) with a yield of 35% (dry weight basis) (Zhang *et al.*, 2007). Recently, Nalinanon *et al.* (2007) studied the use of fish pepsin as the aid to increase the yield of collagen extracted from fish skin. Addition of bigeye snapper pepsin at a level of 20 kUnits/g skin resulted in the increased content of collagen extracted from bigeye snapper skin. The yields of collagen from bigeye

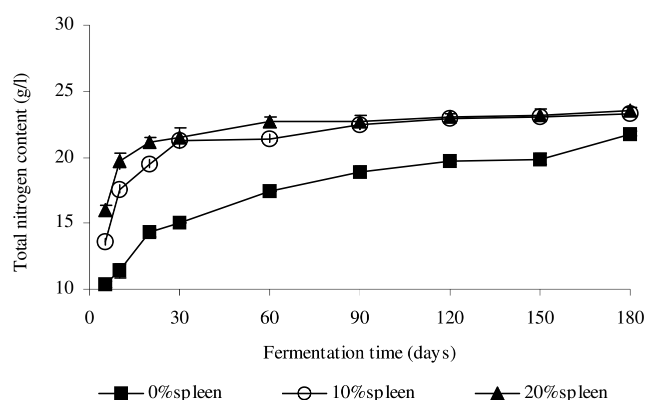

Figure 7. Total nitrogen content of fish sauce samples produced from sardine added with different levels of skipjack tuna spleen during fermentation for 180 days (Klomklao *et al.*, 2006c).

snapper skin extracted for 48 h with acid and with bigeye snapper pepsin were 5.31 and 18.74% (dry basis), respectively. With pre-swelling in acid for 24 h, collagen extracted with bigeye snapper pepsin at a level of 20 kUnits/g skin for 48 h had the yield of 19.79%, which was greater than that of collagen extracted using porcine pepsin at the same level (13.03%) (Nalinanon *et al.*, 2007).

3. Conclusion

Fish digestive proteinases are unique when compared to homologous proteinases from mammals and also show different properties among fish species. These differences are chiefly related to the great range of environmental conditions in which these organisms must cope. Fish digestive proteinases can be very useful tools in the food industry owing to two salient properties: thermal instability and high activity at low processing temperature.

References

Aranishi, F., Ogata, H., Hara, K., Osatomi, K. and Ishihara, T. 1997. Purification and characterization of cathepsin L from hepatopancreas of carp (*Cyprinus carpio*) Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 118, 531-537.

Arunchalam, K. and Haard, N.F. 1985. Isolation and characterization of pepsin isoenzymes from polar cod (*Boreogadus saida*). Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 80, 467-473.

Asgeirsson, B. and Bjarnason, J.B. 1991. Structural and kinetic properties of chymotrypsin from Atlantic cod (*Gadus morhua*). Comparison with bovine trypsin. Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 9, 327-335.

An, H., Peters, M.Y. and Seymour, T.A. 1996. Roles of endogenous enzymes in surimi gelation (Review). Trends in Food Science and Technology. 7, 321-327.

Benjakul, S. and Morrissey, M.T. 1997. Protein hydrolysates from Pacific whitting solid wastes. Journal of Agricultural and Food Chemistry. 45, 3423-3430.

Brewer, P., Helbig, N. and Haard, N.F. 1984. Atlantic cod pepsin. Characterization and use as a rennet substitute. Canadian Institute of Food Science and Technology Journal. 17(1), 38-43.

Cao, M.J., Osatomi, K., Suzuki, M., Hara, K., Tachibana, K. and Ishihara, T. 2000. Purification and characterization of two anionic trypsin from the hepatopancreas of carp. Fisheries Science. 66, 1172-1179.

Cano-Lopez, A., Simpson, B.K. and Haard, N.F. 1987. Extraction of carotenoprotein from shrimp processing wastes with the aid of trypsin from Atlantic cod. Journal of Food Science. 52, 503-506.

Castillo-Yanez, F.J., Pacheco-Aguialr, R.P., Garcia-Carreno, F.L. and Toro, M.A.N.D. 2004. Characterization of acidic proteolytic enzymes from Monterey sardine (*Sardinops sagax caerulea*) viscera. Food Chemistry. 85, 343-350.

Castillo-Yanez, F.J., Pacheco-Aguialr, R.P., Garcia-Carreno, F.L. and Toro, M.A.N.D. 2005. Isolation and characterization of trypsin from the pyloric ceca of Monterey sardine *Sardinops sagax caerulea*. Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 140, 91-98.

Castillo-Yanez, F.J., Pacheco-Aguialr, R.P., Garcia-Carreno, F.L., Toro, M.A.N.D. and Lopez, M.F. 2006. Purification and biochemical characterization of chymotrypsin from the viscera of Monterey sardine (S*ardinops sagax caerulea*). Food Chemistry. 99, 252-259.

Chaveesuk, R., Smith, J.P. and Simpson, B.K. 1993. Production of fish sauce and acceleration of sauce fermentation using proteolytic enzymes. Journal of Aquatic Food Product Technology. 2(3), 59-77.

Chong, A.S.C., Hashim, R., Chow-Yang, L. and Ali, A.B. 2002. Partial characterization and activities of proteases from the digestive tract of discus fish (*Symphysodon aequifasciata*). Aquaculture. 203, 321-333.

Clarks, J., Macdonald, N.L. and Stark, J.R. 1985. Metabolism in marine flatfish. III. Measurement of elastase activity in the digestive tract of dover sole (*Solea solea L*). Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 91, 677-684.

De Vecchi, S. and Coppes, Z.L. 1996. Marine fish digestive proteases in food industry and its relevance to the South-West Atlantic region. Journal of Food Biochemistry. 10, 193-214.

Dimes, L.E., Garcia-Carreno, F.L. and Haard, N.F. 1994. Estimation of protein digestibility: III. Studies on the digestive enzymes from the pyloric ceca of rainbow trout and salmon. Comparative Biochemistry and Physiology A. 109, 349-360.

Faid, M., Zouiten, A., Elmarrakchi, A. and Achkari-Begdouri, A. 1997. Biotransformation of fish waste into stable feed ingredient. Food Chemistry. 60, 13-18.

Frokjaer, S. 1994. Use of hydrolysates for protein supplementation. Food Technology. 48(4), 86-88.

Garcia-Carreno, F.C. and Haard, N.F. 1993. Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. Journal of Food Biochemistry. 17, 97-113.

Garcia-Carreno, F.C. and Hernandez-Cortes, P. 2000. Use of protease inhibitors in seafood products. *In* Seafood Enzymes: Utilization and Influence on Postharvest Seafood Quality, N.F. Haard and B.K. Simpson editors. Marcel Dekker, New York., pp. 531-540.

Gildberg, A. 2001. Utilization of male Arctic capelin and Atlantic cod intestines for fish sauce production-evaluation of fermentation conditions. Bioresource Technology. 76, 119-123.

Gildberg, A. and Raa, J. 1983. Purification and characteriza-

tion of pepsins from the Arctic fish capelin (*Mallotus villosus*). Comparative Biochemistry and Physiology A. 75, 337-342.

Gildberg, A., Olsen, R.L. and Bjannasson, J.B. 1990. Catalytic properties and chemical composition of pepsin from Atlantic cod (*Gadus morhua*). Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 69, 323-330.

Gudmundsdottir, A., Gudmundsdottir, E., Oskarsson, S., Bjanrnason, J.B., Eakin, A.K. and Craik, C.S. 1993. Isolation and characterization of cDNAs from Atlantic cod encoding two different forms of trypsinogen. European Journal of Biochemistry. 217, 1091-1097.

Haard, N.F. 1986. Characterization with casein and milk substrate and influence of sepharose immobilization on salt activation, temperature characteristics and milk clotting reaction. Journal of Food Science. 51, 313-316.

Haard, N.F. 1990. Enzymes from myosystems. Journal of Muscle Foods. 1, 293-338.

Haard, N.F. 1992. A review of protolytic enzymes from marine organisms and their application in the food industry. Journal of Aquatic Food Product Technology. 1(1), 17-35.

Haard, N.F. 1994. Protein hydrolysis in seafoods. *In* Seafood chemistry. Processing Techonology and Quality, F. Shahidi and J.R. Botta, editors. Chapman & Hall, New York, pp.10-33.

Hermodson, M.A., Ericsoon, L.H., Neurath, H. and Walsh, K.A. 1973. Determination of the amino sequence of porcine trypsin by sequenator analysis. Biochemistry. 12, 3146-3153.

Heu, M.S., Kim, H.R. and Pyeun, J.H. 1995. Comparison of trypsin and chymotrypsin from the viscera of anchovy (*Engraulis japonica*). Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 112, 557-567.

Javeed, A., and Mahendrakar, N.S. 1996. Autolysis and rancidity development in tropical freshwater fish viscera during fermentation. Bioresource Technology. 58, 247-251.

Kishimura, H., Hayashi, K., Miyashita, Y. and Nonami, Y. 2005. Characteristics of two trypsin isozymes from the viscera of Japanese anchovy (*Engraulis japonica*). Journal of Food Biochemistry. 29, 459-469.

Kishimura, H., Tokuda, Y., Klomklao, S., Benjakul, S. and Ando, S. 2006a. Enzymatic characteristics of trypsin from pyloric ceca of spotted mackerel (*Scomber Australasicus*). Journal of Food Biochemistry. 30, 466-477.

Kishimura, H., Hayashi, K., Miyashita, Y. and Nonami, Y. 2006b. Characteristics of trypsins from the viscera of true sardine (*Sardinops melanostictus*) and the pyloric ceca of arabesque greenling (*Pleuroprammus azonus*). Food Chemistry. 97, 65-70.

Klomklao, S., Benjakul, S. and Visessanguan, W. 2004. Comparative studies on proteolytic activity of spleen extracts from three tuna species commonly used in Thailand. Journal of Food Biochemistry. 28, 355-372.

Klomklao, S., Benjakul, S., Visessanguan, W., Simpson, B.K. and Kishimura, H. 2005. Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems. Process Biochemistry. 40, 3061-3067.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K. 2006a. Purification and characterization of trypsin from the spleen of tongol tuna (*Thunnus tonggol)*. Journal of Agricultural and Food Chemistry. 54, 5617-5622.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B.K. and Saeki, H. 2006b. Trypsins from yellowfin tuna (*Thunnus albacores*) spleen: Purification and characterization. Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 144, 47-56.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K. 2006c. Effects of the addition of spleen of skipjack tuna (*Katsuwonus pelamis*) on the liquefaction and characteristics of fish sauce made from sardine (*Sardinella gibbosa*). Food Chemistry. 98, 440-452.

Klomklao, S., Kishimura, H., Yabe, M. and Benjakul, S. 2007a. Purification and characterization of two pepsins from the stomach of pectoral rattail (*Coryphaenoides pectoralis*). Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology B. 147, 682-689.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K. 2007b. Purification and characterization of trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis)*. Food Chemistry. 100, 1580-1589.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K. 2007c. Extraction of carotenoprotein from black tiger shrimp shell with the aid of bluefish trypsin. Journal of Food Biochemistry. Accepted.

Kurbanoglu, E.B. and Algur, O.F. 2002. The influence of ram horn hydrolyzate on the crop yield of the mushroom *Agaricus bisporus*. Scientia Horticulturae. 94, 351-357.

Lahl, W.J. and Braun, S.D. 1994. Enzymatic production of protein hydrolysates for food use. Food Technology. 58(10), 68.

Male, R., Lorens, L.B., Smalas, A.O. and Torrissen, K.R. 1995. Molecular cloning and characterization of anionic and cationic variants of trypsin from Atlantic salmon. European Journal of Biochemistry. 232, 677-685.

Martinez, A., Olsen, R.L. and Serra, J.L. 1988. Purification and characterization of two-trypsin-like enzymes from the digestive tract of anchovy, *Engraulis*

encrasicholus. Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 91, 677-684.

Mihalyi, E. 1978. Application of Proteolytic Enzymes to Protein Structure Studies. CRC Press, Boca Raton, Florida, U.S.A.

Nagai, T. and Suzuki, N. 2002. Preparation and partial characterization of collagen from paper nautilus (*Argonauta argo*, Linnaeus) outer skin. Food Chemistry. 76, 149-153.

Nagai, T., Araki, Y. and Suzuki, N. 2002. Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). Food Chemistry. 78, 173-177.

Nagai, T., Yamashita, E., Taniguchi, K., Kanamori, N. and Suzuki, N. 2001. Isolation and characterization of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*). Food Chemistry. 72, 425-429.

Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2007. Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priccanthus tayenus*). Food Chemistry. 104, 593-601.

Noda, M. and Murakami, K. 1981. Studies on proteinases from the digestive organs of sardine. Purification and characterization of two acid proteinases from the stomach. Biochimica et. Biophysica Acta. 658, 27-32.

Nissen, J.A. 1993. Proteases. In Enzymes in Food Processing, T. Nagodawithana and G. Reed, editors. Academic Press, Inc. New York, pp. 159-203.

Pangkey, H., Hara, K., Tachibana, K., Cao, M.J., Osatomi, K. and Ishihara, T. 2000. Purification and characterization of cathepsin S from hepatopancreas of carp *Cyprinus carpio*. Fisheries Science. 66, 1130-1137.

Reid, R.G.B. and Rauchert, K. 1976. Catheptic endopeptidases and protein digestion in the horse clam *Tresus capax* (Gould). Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 54, 467-472.

Sanchez-Chiang, L. and Ponce, O. 1981. Gastricsinogens and Gastricsins from *Merluccius gayi*-purification and properties. Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 68, 251-257.

Sanchez-Chiang, L., Cisternas, E. and Ponce, O. 1987. Partial purification of pepsins from adult and juvenile salmon fish (*Oncorrhynchus keta*). Effect on NaCl on proteolytic activities. Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 87, 793-797.

Shahidi, F., Han, X.Q. and Synowiecki, J. 1995. Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). Food Chemistry. 53, 285-293.

Shamsuzzaman, K. and Haard, N.F. 1984. Characterization of a chymosin-like enzyme from the gastric mucosa of harp seal, *Pagophilus groenlandicus*. Canadian Journal of Biochemistry and Cell Biology. 62, 699-708.

Simpson, B.K. 2000. Digestive proteinases from marine animals. *In* Seafood Enzymes: Utilization and Influence on Postharvest Seafood Quality, N.F. Haard and B.K. Simpson, editors. Marcel Dekker, New York., pp. 531-540.

Simpson, B.K. and Haard, H.F. 1987. Cold-adapted enzymes from fish. *In* Food Biotechnology, D. Knorr, editor. Marcel Dekker, New York., pp. 495-528.

Sivakumar, P., Sampath, P. and Chandrakasan, G. 1999. Collagenolytic metalloprotease (gelatinase) from the hepatopancreas of the marine carb, *Scylla serrata*. Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 123, 273-279.

Stefansson, G. 1988. Enzymes in the fishing industry. Food Technology. 42(3), 64-65.

Stefansson, G. and Steingrimsdottir, V. 1990. Application of enzymes for fish processing in Iceland: Present and Future Aspects. *In* Advances in Fisheries Technology and Biotechnology for Increased Profitability, N.M. Voigt and H.R. Botta editors. Technology Publishing Co., Lancaster, Pennsylvania., pp. 237-250.

Stom, T. and Eggum, B.O. 1981. Nutritional value viscera silage. Journal of the Science of Food and Agriculture. 32, 115-120.

Torrissen, K.R. 1984. Characterization of proteases in the digestive tract of Atlantic salmon (*Salmo salar*) in comparison with rainbow trout (*Salmo gairdneri*). Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 77, 669-674.

Vecht-Lifshitz, S.E., Almas, K.A. and Zomer, E. 1990. Microbial growth on peptones from fish industrial wastes. Letters in Applied Microbiology. 10, 183-186.

Walsh, K.A. 1970. Trypsinogens and trypsins of various species. Methods in Enzymology. 19, 41-63.

Wasserman, B.P. 1990. Evolution of enzymes technology: Progress and prospects. Food Technology. 44(4), 118.

Wray, T. 1988. Fish Processing: New uses for enzymes. Food Manufacture. 63(3), 64-65.

Whitaker, J.R. 1994. Classification and nomenclature of enzymes. *In* Principles of Enzymology for the Food Sciences, Marcel Dekker, New York., pp. 367-385.

Xu. R.A., Wong, M.L., Rogers, M.L. and Fletcher, G.C. 1996. Purification and characterization of acidic proteases from the stomach of the deep water finfish orange roughy (*Hoplostethus atlanticus*). Journal of Food Biochemistry. 20, 31-48.

Zeef, A.H. and Dennison, C. 1988. A novel cathepsin from the mussel (*Perna perna Linne*). Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology. 90, 204-210.

Zhang, Y., Liu, W., Li, G., Shi, B., Miao, Y. and Wu, X. 2007. Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*). Food Chemistry. 103: 906-912.