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.

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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,
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 several 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., 1996), 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).

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 ractoralis) 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 catchingus) and tongol tuna (Thunnus tonggol) (Klomklao et al., 2006a). 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).

Figure 1. Action of endopeptidases and exopeptidases on protein structure (An et al., 1996).
villosus) (Gildberg and Raa, 1983), polar cod (Boreogadus saida) (Arunchalam and Haard, 1985) sardine (Sardinops melanostica) (Noda and Murakami, 1981) and Monterey 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 morhua) 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).

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 aszymogens 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
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 isozyms were purified and characterized from the gastric juices of hake (Sanchez-Chiang and Ponce, 1981). The latter property would appear to distinguish the gastricsins from pepsins (Simpson, 2000). The optimum pH for the hydrolysis of hemo-globin 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 optimum pH and temperature for TAME hydrolysis of gastricsin were 8.5 and 60°C, respectively (Figure 5).

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

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 aspartyl proteinases that have many properties in common with other enzymes in the family of serine proteinases, specifically hydrolyze proteins of a serine residue, together with an imidazole group and aspartyl carboxyl group in their catalytic sites (Simpson, 2000; Klomklao et al., 2000). 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). Trypsin from yellowfin tuna (Thunnus albacores) spleen. Trypsins A and B exhibited the maximal activity at 55 and 65°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 melano-stictus) and pyloric ceca of arabesque greenling (Pleuror-hamus 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, Sephade 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).

Trypsin 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
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 designing 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).

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

<table>
<thead>
<tr>
<th>Source of trypsin</th>
<th>N-terminal sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skipjack tuna</td>
<td>IVGGYECQAHSQPHQVSLNS</td>
<td>Klomklao et al. (2007b)</td>
</tr>
<tr>
<td>Yellowfin tuna</td>
<td>IVGGYECQAHSQPHQVSLNA</td>
<td>Klomklao et al. (2006a)</td>
</tr>
<tr>
<td>Tongol tuna</td>
<td>IVGGYECQAHSQPHQVSLNA</td>
<td>Klomklao et al. (2006b)</td>
</tr>
<tr>
<td>True sardine</td>
<td>IVGGYECQAKYSQPWQVSLNS</td>
<td>Kishimura et al. (2006b)</td>
</tr>
<tr>
<td>Japanese anchovy</td>
<td>IVGGYECQAHSQPHTVSLNS</td>
<td>Kishimura et al. (2005)</td>
</tr>
<tr>
<td>Cod</td>
<td>IVGGYECQKBSQAHQVSLNS</td>
<td>Gudmundsdottir et al. (1993)</td>
</tr>
<tr>
<td>Salmon</td>
<td>IVGGYECQKYSQTHQVSLNS</td>
<td>Male et al. (1995)</td>
</tr>
<tr>
<td>Porcine</td>
<td>IVGGYTCAAANSVPYQVSLNS</td>
<td>Hermodson et al. (1973)</td>
</tr>
<tr>
<td>Bovine</td>
<td>IVGGYTCGANTVPYQVSLNS</td>
<td>Walsh (1970)</td>
</tr>
</tbody>
</table>

3) Thiol/Cysteine proteinases

Thiol or cysteine proteinases are a group of endo-
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 organs 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). Metalloproteinases 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

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

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Application</th>
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<tbody>
<tr>
<td>Cereals, baked goods</td>
<td>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</td>
</tr>
<tr>
<td>Egg and egg products</td>
<td>Improve quality of dried products</td>
</tr>
<tr>
<td>Meats</td>
<td>Tenderization; recover protein from bones; hydrolysis of blood proteins</td>
</tr>
<tr>
<td>Fish</td>
<td>Fish protein hydrolysates, viscosity reduction, skin removal, roe processing</td>
</tr>
<tr>
<td>Pulses</td>
<td>Tofu; soy sauce; protein hydrolysis; off-flavor removal soy milk</td>
</tr>
<tr>
<td>Dairy</td>
<td>Cheese curd formation; accelerate cheese aging; rennet puddings</td>
</tr>
<tr>
<td>Brewing</td>
<td>Fermentation and filtration aid; chill proofing Clarification; decrease foaming, promote malolactic fermentation</td>
</tr>
<tr>
<td>WineCoco</td>
<td>Facilitate fermentation for chocolate production</td>
</tr>
</tbody>
</table>
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 proteases 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 proteases 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-adapted 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 proteases 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.

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