

Extraction, Purification and Characterization of Fish Pepsin: A Critical Review

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Abstract

The current disposal of fish processing waste is causing environmental problems that have become an issue of public concern. Fish waste could be utilized for production of animal feed, biodiesel, natural pigments, food products and pharmaceuticals. Major attention was also directed to the recovery of valuable biomolecules such as collagen ω -3 fatty acid, trypsin, chymotrypsin and elastase. Among these molecules, pepsin is one of the most beneficial and useful biomolecule that can be recovered from fish wastes. Pepsin is important aspartic proteases with many unique characteristics. It has several industrial applications including collagen extraction, cheese making, fish silage making, fish processing as well as use in medical research. Fish pepsin is primarily present in fish stomach and has a characteristic pH optimum of 2.0 - 4.0, distinct pH stability \leq 6.0, a distinct optimum temperature of 30-55°C and specific thermal stability \leq 40-50°C. Pepstatin A can strongly inhibit the pepsin activity while PMSF, E-64 and EDTA have a negligible impact. SDS, cysteine and aliphatic alcohols, have been identified as effective inhibitors while ATP, molybdate, NaCl, MgCl₂, and CaCl₂ do not inhibit the activity of pepsin. Pepsin is widely applied in collagen extraction, in digestibility therapy, as rennet substitute and. At present, conventional method and innovative method have been developed for fish pepsin recovery. In the conventional method (ammonium sulfate), the partition of pepsinogen (PG) is mainly based on (a) crude enzyme extraction by homogenization and centrifugation, (b) purification of PG by gel filtration and anion exchange chromatography and (c) activation of PG under the acidic condition. The innovative recovery is based on aqueous two-phase system (ATPS). The conventional method is used for high purification while the ATPS is used for partial purification. Enzyme activity and concentration, specific activity (SA), purification factor (PF), molecular weight, and homogeneity, isoelectric point (pI) and are used to assay fish pepsin.

Keywords: Fish; Pepsin; Dimensional structure; pH; Stability; Temperature; Inhibitor; Partition; Conventional methods; Aqueous two phase system; Assay

Introduction

The growing consumer demand for healthy fish products has led to a thriving fish processing industry worldwide. Processing of fish includes: scaling, cutting, filleting, cooking, salting and canning. When fish fillets or cans are produced, a large fraction (30–80%) of fish (flesh, heads, bones, fins, skin, tails and viscera) is left as waste. Fish waste is usually disposed off in landfills or in the sea resulting in environmental problems which underscore the need for a proper utilization of fish wastes for the recovery of valuable products. Fish waste can be utilized as ingredient in animal feed and fertilizer [1,2] or used for the recovery of valuable biomolecules such as collagen [3,4] ω -3 fatty acid [5] trypsin [6,7] chymotrypsin [8] and elastase [9] or the production of biodiesel [10].

Pepsin is an important acidic protease that is widely applied in the hydrolysis of proteins. It is used in collagen extraction [11-13] in gelatin extraction [14] as a rennet substitute [15] and in digestibility therapy [16]. Pepsin has been isolated and investigated from various mammals including: human [17] Japanese monkey [18] pig [19] bovine [20] goat [21] rat [22] and rabbit [23]. Pepsin can also be recovered from fish wastes, especially fish viscera which constitute 5% of fish weight [24]. Isolation and purification of pepsin from fish and fish wastes is less developed. Thus, an efficient and economic process for the isolation and purification of pepsins from fish wastes would bring significant economic and environmental benefits for both the fish processing and pepsin industries.

Structures, Sources and Classification of Pepsin

Pepsin (molecular weight of 36 k Da) is an enzyme in the aspartic protease family which digests proteins. Peptide bonds can be readily

cleaved allowing degradation of proteins under acidic conditions [25]. Pepsin can be found primarily in the gastric juice of the stomach lumen and can be isolated from a variety of species such as human, cattle, sheep, birds and fish [26,27]. The main location of pepsin in mammals is the stomach (both the membrane and the gastric juice) but it can also be found in limited amounts in the blood, muscle and the urine [27]. Fish pepsins are mostly present in the stomach but can also be found in the ovaries of brook trout or the skin of puffer fish [28,29]. There are several types of stomach pepsins, each has a distinct protein structure and enzymatic properties [30].

Pepsin is synthesized and secreted in the gastric membrane in an inactive state called pepsinogen (PG) (molecular weight of 40kDa). Compared with pepsin, pepsinogen contains an additional 44 amino acids and is stable in neutral and weak alkaline environments, but when exposed to the hydrochloric acid (HCl) present in gastric juice (pH of 1.5 – 2.0), the 44 amino acids are proteolytically removed in an autocatalytic way to activate it to pepsin [31]. Its main role in protein proteolysis is to cleave aromatic amino acids (such as phenylalanine and tyrosine) from the N-terminus of proteins [31].

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The three-dimensional structures of pepsin and pepsinogen from fish species are shown in Figure 1. While mammal pepsin has been completely sequenced, fish pepsin has not been extensively explored. The first proposed three-dimensional structure for cod pepsin was identical to porcine pepsin in domain location and amino acid formation (57.7% sequence identity) [32,33]. Cod pepsin is reported as a single chained protein (a monomer) of two similar folded domains separated by a deep cleft [32]. The catalytic site of pepsin is formed at the junction of the domains and contains two aspartic acid residues, Asp 32 and Asp 215 in each domain [33,34]. In the catalysis of pepsin, water molecule helps between the active carboxyls and Asp 215 and Asp 32 becomes negatively and positively charged individually to break peptide bonds in proteins [35,36].

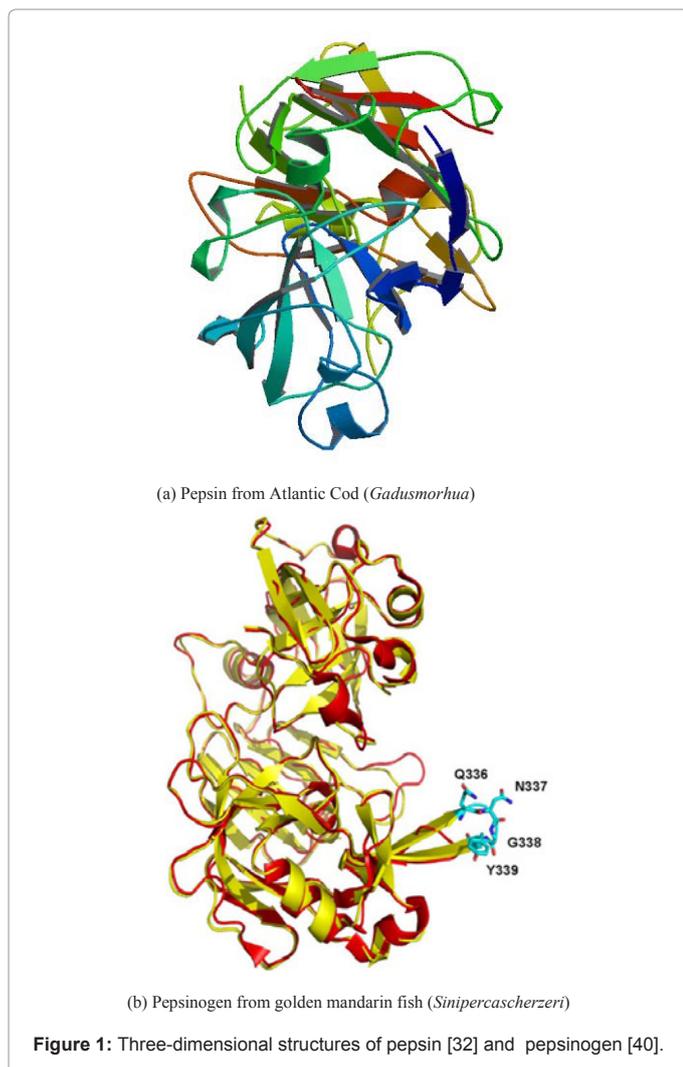
The nomenclature and classification of pepsin depends primarily on the enzyme characteristics including activity, stability and kinetics [2,37-39] and properties of enzyme including aspartate residues for the catalysis of substrates, protein degradation and the tertiary structure [36,40-42]. Mammalian pepsinogens (PGs) are divided into two groups: (a) a major group which includes pepsinogen A (PG-A), pepsinogen C (PG-C, or progastricsin) and pepsinogen Y (PG-Y or prochymosin) and (b) a minor group which includes pepsinogen B (PG-B) and pepsinogen F (PG-F) [23,41,43,44]. Classification of fish

pepsinogen varies among researchers. Some researchers followed the mammal pepsin classification system and divided fish pepsinogens to PG-A, PG-B and PG-C [45-48] while others divided fish pepsinogens to PG-I, PG-II, PG-III and PG-IV [30,49-52]. However, fish PGs have distinct characteristics from mammalian PGs [53] including higher pI [54] higher pH optimum and Michaelis constant [55] lower optimum temperature and thermal stability [56] than mammalian PGs.

Fish PGs (or pepsins) have been purified and characterized in various types of fishes including Arctic capelin [48] rainbow trout [57] Atlantic cod [58] boliti fish [2] Antarctic rock cod [59] sea bream [51] African coelacanth [49] Mandarin fish [60] smooth hound [50] orange-spotted grouper [47] albacore tuna [61] and European eel [52]. The amounts and types of PGs vary among fish species as different fish species have different PG isoforms in the stomachs. Bougateg et al. [50] isolated only one type of PGs from the stomach of smooth hound and obtained 7.33mg of PGs/100g stomach. Gildberg and Raa [48] isolated two pepsins (pepsins I and II) from the Arctic capelin and obtained 13.9 and 0.5 mg per 100 gastric membranes, respectively. Tanji et al. [49] isolated three types of PGs (PGs I, II and III) from the stomach of African coelacanth and obtained 12.1, 8.57 and 16.7 mg per 100g stomach mucosa, respectively. Wu et al. [52] isolated three types of PGs (PGs I, II and III) from the stomach of European eel and obtained 3.63 mg per 100g stomachs. Zhou et al. [51] found four types of PGs in sea bream stomachs with a total amount of 35.5 mg per 100 g stomach. Zhou et al. [60] isolated four PGs from the mucosa of mandarin fish with a total amount of 36.6 mg/100g stomach. However, among all the fish pepsins, pepsins from cod and tuna are the most investigated.

Cod pepsins have been studied and sequenced by many researchers [39,46,59,61-63]. Gildberg [53] reported that cod (cold water fish) has pepsins which are more active at low temperature than those from warm water fishes. He also indicated that a high content of pepsin (50-100 mg pepsin per 100 g cod stomach) is found in Atlantic cod. Bjelland et al. [64] found two different types of pepsins in cod (Pepsin I and Pepsin II) which are similar to mammalian pepsins in structure but are more active at low temperatures and weak acid conditions and more easily inactivated by moderate heating as cod is more adaptable to cold temperature compared to mammals which are usually live in warm habitats. Gildberg et al. [46] indicated that pepsin I functions under relatively weak acidic condition ($p^H=4.0$) while pepsin II has a similar pH optimum to mammalian pepsin and more active in strong acidic condition ($p^H=2.0$). Gildberg [56] reported higher content of basic amino acids present in fish pepsin I and explained the difference. Brier et al. [59] reported similar results with these two types of pepsins and noted that they have greater sensitivity to inhibitors such as pepstatin, which is probably attributed to changes in the sequence of fish pepsin near the active site.

Albacore tuna have only one type of pepsin [65]. Tongol tuna contains two isoforms of pepsin while only one type of pepsin was found in skipjack tuna stomach [66]. Nalinanon et al. [65] indicated that pepsin in tuna has a molecular weight and an active temperature that are different from those of bovine pepsin. Tanji et al. [67] detected three PGs (PG-I, PG-II and PG-III) in Pacific blue fin tuna, which contain a greater number of basic residues than mammalian PGs and are found to be most active at a p^H of 2.5. Tuna pepsins I and II can be greatly inhibited by the protease inhibitor pepstatin A [65,67]. Tanji et al. [44] indicated that pepsins in blue fin tuna have unique properties and functions which are diverse than pepsins from other fish species. Pepsin from yellow fin tuna has an optimum temperature of 45°C, an



optimum p^H of 2.5 and crystal structure, specificity, alkaline stability and other properties which are different from swine pepsins [68].

Factors Affecting the Activity of Pepsins

The activity of pepsin measured by its ability to hydrolyze proteins is influenced by p^H, temperature and inhibitors.

pH

Both the optimum pH (the pH value giving the highest enzymatic activity) and pH stability (the pH range giving good enzyme stability) have significant effects on the activity of fish pepsin. When the pH deviates from the optimum value, the activity of pepsin drops. El-Beltagy et al. [2] reported that pepsins I and II from boliti fish had the same optimum pH of 2.5 and deviation from that pH value resulted in reduced activity (Figure 2). A summary of optimum pH values for pepsin from different fishesis displayed in Table 1. In general, if the fish has more than one type of pepsin, their optimum pH will be similar. However, this fact does not apply to some fishes such as Arctic capelin [48]. Generally, pepsin stability is mentioned at lower pH values. Pepsin is a type of acidic protease and depression of its stability is attributed to the denaturation of proteins above the pH of 6.0 [61]. Klomklao et al. [45] studied the effect of pH on the pectoral rattail pepsins and found that pepsin A and pepsin B were stable in the pH range of 2-6 and their activities dropped dramatically when the pH exceeded 6. Nalinanon et al. [65] found albacore tuna pepsin was stable within the pH range of 2-5. Similar results were reported with several fish pepsins [48,64,69-71].

Temperature

Temperature has a great influence on the activity of fish pepsin. Like pH, the optimum temperature and thermal stability range are very important. A summary of optimum temperature for fish pepsin is displayed in Table 2. The optimum temperature of fish pepsin ranges from 30 to 55°C. When the temperature deviates from the optimum value, the activity of pepsin drops. The optimum temperature of fish pepsin depends greatly on fish species (cold or warm water species) [30,64,72,73]. Fishes from cold water habitats have lower optimum temperature than those from warm water habitat [61,64,70]. The activities of pepsins I and II from warm water sardine had relatively

Enzyme	Identified Species	Optimum p ^H	Reference
Pepsin	Smooth hound	2.0	Bougatef et al. [50]
Pepsin I	Arctic capelin	3.7	Gildberg and Raa [48]
Pepsin II	Arctic capelin	2.5	Gildberg and Raa [48]
Pepsin I	European eel	3.5	Wu et al. [52]
Pepsin II	European eel	2.5	Wu et al. [52]
Pepsin III	European eel	2.5	Wu et al. [52]
Pepsin	Greenland cod	3.5	Squires et al. [61]
Pepsin	Atlantic cod	3.5	Haard [62]
Pepsin	Arctic cod	3.0	Haardet al. [72]
Pepsin I	Atlantic cod	3.5	Gildberg [58]
Pepsin II	Atlantic cod	3.0	Gildberg [58]
Pepsin	palometa	3.0-3.5	Pavlskoet al. [73]
Pepsin I	Sea bream	3.0	Zhou et al. [51]
Pepsin II	Sea bream	3.5	Zhou et al. [51]
Pepsin III	Sea bream	3.5	Zhou et al. [51]
Pepsin IV	Sea bream	3.5	Zhou et al. [51]
Pepsin	Albacore tuna	2.0	Nalinanon et al. [65]
Pepsin I	Mandarin fish	3.5	Zhou et al. [60]
Pepsin II	Mandarin fish	3.5	Zhou et al. [60]
Pepsin III	Mandarin fish	3.5	Zhou et al. [60]
Pepsin IV	Mandarin fish	3.5	Zhou et al. [60]
Pepsin I	African coelacanth	2.0	Tanji et al. [49]
Pepsin II	African coelacanth	2.0	Tanji et al. [49]
Pepsin III	African coelacanth	2.5	Tanji et al. [49]

Table 1: The optimum p^H of pepsin from different fishes.

high temperature optima of 40°C and 55°C, respectively [70] while pepsins I and II from cold water Arctic capelin showed the greatest activity at 38 and 43°C, respectively [48]. Every type of pepsin has unique thermal properties and enzymes from cold water species have relatively low optimum temperatures. Simpson and Haard [74] stated that cold water fish enzymes have a low Arrhenius activation energy which explains their low optimal temperatures and high heat liability compared with warm water counterparts. Haard [54] indicated that genes in different fish species account for the diversity of the enzymic characteristics.

The upper temperature limit for pepsin stability depends on the type of fish. However, pepsins from cold water species are quite susceptible to higher temperatures [49,57-59]. Nalinanon et al. [65] reported that pepsin from albacore tuna (a type of warm water fish that can adapte to waters with 13.5-25.2°C) retained its stability at temperatures ranging from 20 to 50°C and the stability of pepsin decreased significantly when the temperature increased above 50°C. Boliti fish remained stable below 60°C [2] and Monterey sardine was active below 45°C [71]. The upper temperature limit for pepsins from other warm water species such as dogfish, pectoral rattail and smooth hound are 50°C, 40°C and 50°C, respectively [45,50,75]. The sharp decrease of thermal stability is attributed to the denaturation of pepsin and destruction of its structure above temperature upper limit [62,65,76].

Inhibitors

Pepstatin A (a typical aspartic proteinase inhibitor) can combine with fish pepsin and prevents the binding of the enzyme to substrate,

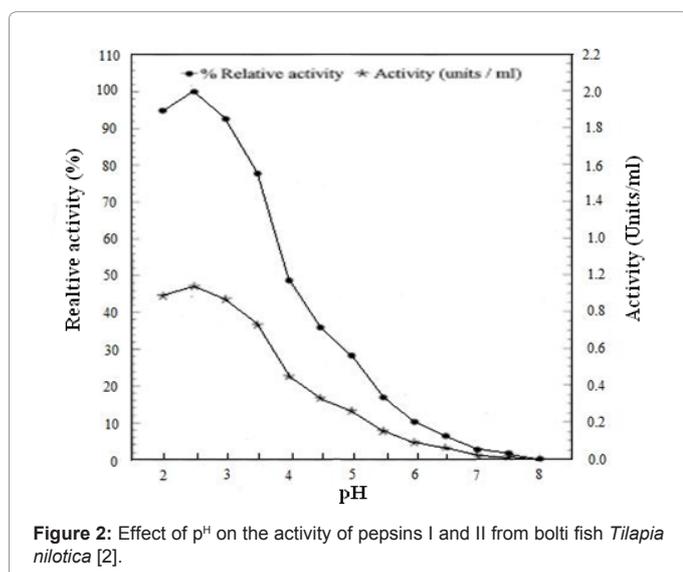


Figure 2: Effect of p^H on the activity of pepsins I and II from boliti fish *Tilapia nilotica* [2].

thereby resulting in a complete inhibition of its activity [36,51,52,77]. Pepstatin A is a peptide isolated from several species of actinomycetes, such as *Streptomyces spp* [78]. It has one of the lowest known inhibition constant ($K_i = 45 \text{ pM}$) for pepsin [77,79]. Zhou et al. [51] studied the inhibitory effect of pepstatin on sea bream fish pepsin and found that the pepstatin formed a complex with Pepsins I, II, III and IV at different pepstatin: pepsin ratios. This molar pepstatin to pepsin ratio played an essential role in the inhibition. According to Bougatef et al. [50] a ratio of about 1:1 gave an entire inhibition of mandarin fish pepsins III and IV while a ratio of 10:1 was necessary to inhibit pepsins I and II. Pepsin from smooth hound was found to be completely inhibited with a molar pepstatin: pepsin ratio of 16:1. Molar pepstatin: pepsin ratios for bluefin tuna, two pepsins from bullfrog and two pepsins from Antarctic rock cod were found to be 17:1, 17:1 and 1:1, respectively [59,67,80].

Not every protease inhibitor has an inhibitory effect on pepsin. It has been shown that typical inhibitors such as phenyl methyl sulfonyl fluoride (PMSF) which is a serine proteinase inhibitor; L-3-carboxy-trans-2, 3-epoxy-propionyl-L-leucine-4-guanidino-butylamide (E-64) which is a cysteine proteinase inhibitor and Ethylene diamine tetra acetic acid (EDTA) do not have any inhibitory effect on pepsins [45,50-52,71]. However, some chemicals have inhibitory effects on the activity of fish pepsin. Sodium dodecyl sulfate (SDS) at 0.05-0.10% (w/v) had a strong inhibitory effect on the activity of pepsins from albacore,

skipjack and tongol tunas while cysteine (5-50 mM) showed some inhibitory effects on them [66]. Aliphatic alcohols (methanol, ethanol, amylalcol) inhibit the activity of pepsin at low concentrations. The inhibition of pepsin by iso amyl alcohol occurs at concentration less than 0.09 M [81]. Adenosine tri phosphate (ATP), molybdate, NaCl, MgCl_2 , and CaCl_2 do not have any impact on the activity of pepsins from albacore tuna [65] skipjack tuna and tongol tuna [66] turbot [82] and pectoral rattail [45].

Industrial Application of Pepsins

Collagen extraction

Conventionally, collagen is extracted by an acid-solubilization process (ASP) in which collagen is solubilized in an acid (such as acetic acid) and other non-acid-soluble materials are removed [37]. Since pepsin can break down cross-linkages in the telopeptide regions of collagen without harming to its secondary structure, the use of pepsin in this process can effectively enhance the yield of collagen [11,13,37]. Nagai and Suzuki [3] and Nagai et al. [83] used mammalian pepsin in collagen extraction and found significance increases in collagen yield. Fish pepsins have been used in collagen extraction by several researchers. Nalinanon et al. [13] reported that with addition of the same fish pepsin the yield of collagen from the skin of big eye snapper (*Priacanthustayenus*) increased to 18.7% compared with a

Enzyme	Identified Species	Habitat	Optimum Temperature (°C)	Reference
Pepsin I	Sardine	Warm water	55	Noda and Murakami [70]
Pepsin II	Sardine	Warm water	40	Noda and Murakami [70]
Pepsin I	Arctic capelin	Cold water	38	Gildberg and Raa [48]
Pepsin II	Arctic capelin	Cold water	43	Gildberg and Raa [48]
Pepsin I	European eel	Warm water	40	Wu et al. [52]
Pepsin II	European eel	Warm water	40	Wu et al. [52]
Pepsin III	European eel	Warm water	35	Wu et al. [52]
Pepsin	Greenland cod	Cold water	30	Squires et al. [61]
Pepsin	Arctic cod	Cold water	32	Haardet al. [72]
Pepsin	Polar cod	Cold water	37	Arunchalam and Haard [76]
Pepsin I	Atlantic cod	Cold water	40	Gildberg [58]
Pepsin II	Atlantic cod	Cold water	40	Gildberg [58]
Pepsin	Palometa	Warm water	37	Pavliiskoet al. [73]
Pepsin I	Sea bream	Warm water ^a	45	Zhou et al. [51]
Pepsin II	Sea bream	Warm water	50	Zhou et al. [51]
Pepsin III	Sea bream	Warm water	50	Zhou et al. [51]
Pepsin IV	Sea bream	Warm water	50	Zhou et al. [51]
Pepsin	Albacore tuna	Warm water	50	Nalinanon et al. [65]
Pepsin I	Mandarin fish	Warm water	40	Zhou et al. [60]
Pepsin II	Mandarin fish	Warm water	45	Zhou et al. [60]
Pepsin III	Mandarin fish	Warm water	40	Zhou et al. [60]
Pepsin IV	Mandarin fish	Warm water	45	Zhou et al. [60]
Pepsin I	Pectoral rattail	Warm water	45	Klomklao et al. [45]
Pepsin II	Pectoral rattail	Warm water	45	Klomklao et al. [45]

Table 2: The optimum temperature of pepsin from different fishes.

yield of 5.3% obtained with ASP. Ahmad and Benjakul [84] reported that with the addition of albacore tuna and yellow fin tuna pepsins, the yields of collagen isolated from the skin of unicorn leather jacket (*Aluterus monoceros*) were 8.48% and 8.40%, respectively (which were higher than the 4.19% obtained with ASP). Nalinanon et al. [66] reported that after 12h extraction using pepsins from albacore tuna, skipjack tuna and tongol tuna, 74.48%, 63.81% and 71.95% of collagen were isolated from the skin of thread fin bream (*Nemipterus spp.*), respectively. These values were greater than the yield of 22.45% obtained with ASP.

Medical research

Pepsin is utilized in regulation of digestion, as a dental antiseptic and in treatment of ailment including dyspepsia, gastralgia, obstinate vomiting, infantile diarrhea, aepsia and some cancer [85]. Combined with HCl, pepsin tablets and capsules have been developed to support the digestibility in the gastrointestinal tract as well as to enhance the appetite of patients [86]. Pepsin from porcine stomach is used for treatment of gastric ulcers with bismuth complexes added [87]. Pepsin is also added for better digestibility of proteins in animals feed.

Cheese making

Animal (calf, bovine and porcine sources) rennet is composed of pepsin and chymosin commonly mixed in a ratio of 1:9 and used in commercial cheese making. However, their high costs have forced the industry to find new substitutes and fish pepsin has been regarded as one such promising substitute [62,74,88]. Brewer et al. [88] found cod pepsin to have a higher ability to clot milk than calf chymosin at 15°C and the prepared cheese had good flavour when tested by a sensory panel. Haard [62] stated that cod pepsin has a lower temperature coefficient (1.4) for milk clotting than that of calf rennet (2.2). Tavares et al. [89] indicated that tuna pepsin is effective as a commercial rennet in cheese production within the pH range of 5.5~6.5. Despite these promising results, cheese production based on fish pepsin has not yet been commercialized [15,24].

Fish silage

Fish pepsin can be used in the production of fish silage, a liquefied product of minced fish and fish visera solubilized by endogenous proteolytic enzymes in the presence of an acid [90-93]. Pepsin plays a major role among all enzymes in preparation of silage [53]. It can degrade and liquefy minced raw material quickly into highly nutritious protein hydrolysate [53,86,90,94,95]. Gildberg and Almås [91] indicated that pepsins I and II in cod viscera were effectively used for the production of aqueous phase of cod viscera silage under acidic conditions. Goddard and Al-Yahyai [94] reported that good silage was obtained with fresh minced sardine (as a source of pepsin) and formic and propionic acids mixture.

Fish processing

Fish pepsin can be utilized in gentle processing of some fish raw materials. Pepsin from cold-water species such as Atlantic cod (*Gadus morhua*) and orange roughy (*Hoplostethus atlanticus*) have been used for caviar production from the roe of the same species in New Zealand [69]. Cod pepsin was tested for deskinning of herring [96] and used to descale hake and haddock under weak acidic conditions [58]. At present, Atlantic cod crude pepsin has been commercialized for the descaling of fish [53].

Partitioning of Pepsin

The process of partitioning of fish pepsin involves three steps: (a) extraction of pepsinogen which involves preparation of fish stomach and crude extract, (b) purification of pepsinogen and (c) activation of pepsinogen to pepsin. All these steps are conducted at low temperatures ($\leq 4^\circ\text{C}$) to prevent denaturation of proteins [97]. Purification can be achieved by the conventional method or by the aqueous two-phase system. Three different steps (Figure 3) can be used for purification of pepsinogens (PGs) under the conventional method: ammonium sulphate fractionation, gel filtration or anion exchange chromatography [50,98-100]. However, if high homogeneity of pepsin is not required and a mixture of several types of pepsins from same fish species with a low level of impurities, pepsin can be extracted and purified using the aqueous two-phase system (ATPS) as shown in Figure 4.

Extraction of pepsinogen

Preparation of fish stomach (or mucosa): The preparation of fish stomach involves the separation of the stomach or mucosa (a mucous membrane layer of the stomach containing the glands and the gastric pits which are the source of pepsinogen) from other organs in the fish viscera. The stomachs must be stored at a low temperature (-20°C or -80°C) until used in order to minimize the autolysis of proteases [45,47,50,64,65,67,101]. El-Beltagy et al. [2] and Castillo-Yáñez et al. [71] stored the viscera of boliti fish and Monterey sardine at -20°C before they were used for enzyme extraction. Tanjiet al. [102] stored stomachs of North Pacific bluefin tuna at -80°C before extraction.

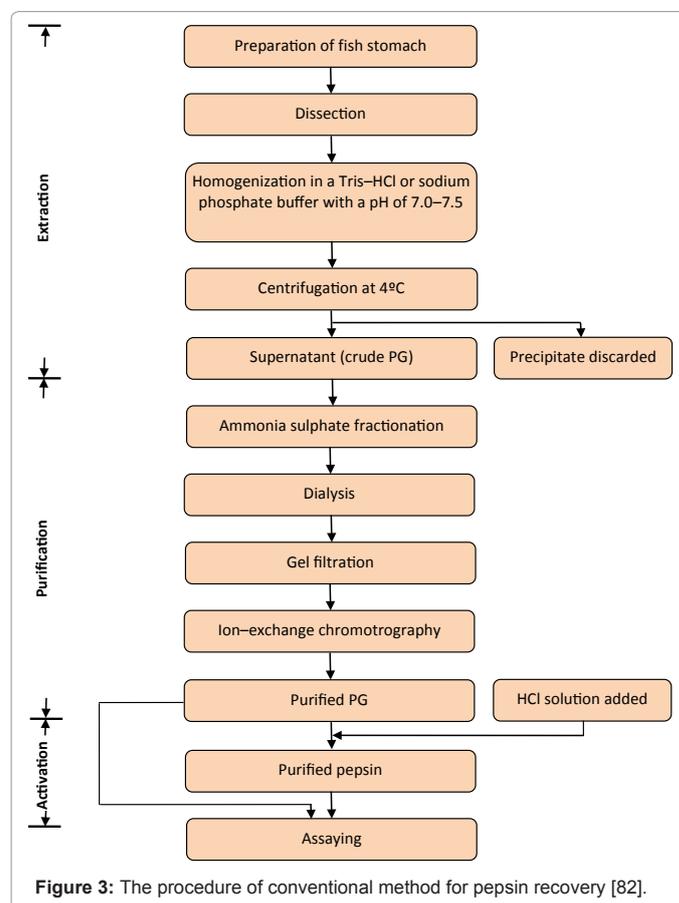
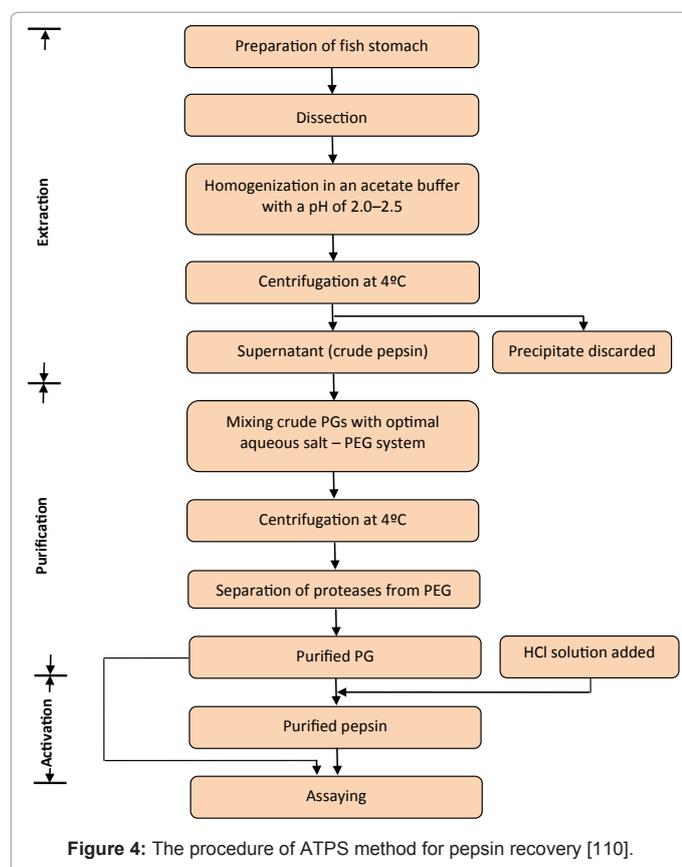


Figure 3: The procedure of conventional method for pepsin recovery [82].



Gildberg and Raa [48] stored whole Arctic capelin at -20°C before thawing and removal of stomachs. Feng et al. [47] stored whole orange-spotted groupers at -80°C before removal of specific organs.

Preparation of crude extract: A variety of methods have been used to isolate the enzymes from stomach mucosa cells, all of which involve three steps: (a) dissection and homogenization of samples to disrupt the cells to release the PGs, (b) solubilization of PGs in an appropriate buffer to control the pH and maintain PGs stable (c) centrifugation of the mixture to remove the cell residues from PGs. The temperature plays a significant role in crude enzyme recovery and the extraction procedure should be conducted at a low temperature ($0-4^{\circ}\text{C}$) to minimize protein denaturation as well as the autolysis of enzymes [103].

The use of buffer solutions is important in PG extraction and their pH must be controlled at 7.0–7.5 to keep the enzymes stable. Sodium phosphate buffer, Tris-HCl buffer and distilled water are the most commonly used solvents to extract the crude PG. Twining et al. [57] Pavlisko et al. [73] Zhou et al. [51] Tanji et al. [49] and Wu et al. [52] used sodium phosphate buffers having a pH in the range of 7.0–7.5 to solubilize the enzymes. Brewer et al. [88] Squires et al. [61] Chong et al. [104] Brier et al. [59] and Bougateg et al. [50] used a Tris-HCl buffer with a pH of 7.5 to solubilize the enzymes. Distilled water was, also, used by Bjelland et al. [64], Gildberg [58], Castillo-Yáñez et al. [71] and Nalinanon et al. [65] as an enzyme solvent.

Doonan [103] stated that the mixing and homogenization operations applied in the preparation of crude extract gave sufficient disruption of mucosa cells and ensured that the released proteins were

evenly dissolved in the buffer. Two types of homogenizations have been reported in the literature. Most researchers homogenize the stomachs in a buffer and as such directly release PGs into the buffer solution [50,57,70,71,82]. Others homogenize the sample in a liquid nitrogen, freeze-dry it to yield a fine enzyme powder and then solubilize this powder in a buffer [47,65]. Another method for the preparation of PG powder involves homogenizing the stomachs in acetone at -20°C and air-drying it at ambient temperature to obtain a fine stomach powder [2,45,105].

Centrifugation is used to remove the insoluble materials from the mixture of enzymes and mucosaresidues and to separate the crude PG. The condition of centrifugation is denoted by the centrifugation field (g), a term used to describe the centrifugation force. Tanji et al. [49] centrifuged a homogenized suspension at 20,000 g for 20 min at 4°C to separate the crude albacore tuna enzyme. Wu et al. [52] acquired crude PGs of European eels by centrifugation at 18,000g for 90 min. Several researchers [45,48,57,71] recommended centrifugation at 15,000g for 30 min or 20,000g for 20 min.

Conventional purification methods of pepsinogen

Ammonia sulfate fractionation: Ammonium sulfate fractionation (ASF) serves as the principal approach to purify enzymes [106]. A mixture of proteins in the supernatant is typically obtained in the centrifugation step, which can be precipitated and separated in $(\text{NH}_4)_2\text{SO}_4$ solution. Usually, two saturations of $(\text{NH}_4)_2\text{SO}_4$ solution are applied to achieve purification. Lower saturation (20-30%) serves to precipitate unwanted proteins, which can be discarded through centrifugation while higher saturation (60-70%) is applied to the supernatant for pooling of desired proteins [97,107]. Wu et al. [52] and Bougateg et al. [50] used 20-60% saturation to achieve high purification of European eel and smooth hound PGs, respectively. Gildberg and Raa [48] and Klomkiao et al. [45] used 30–70% saturations to purify PGs from Arctic capelin and pectoral rattail, respectively. El-Beltagy et al. [2] used 40–60% saturation for the purification of Bolti fish PGs. After this step, dialysis or diafiltration is performed to remove the $(\text{NH}_4)_2\text{SO}_4$ from the enzyme precipitates [107].

Ion-exchange chromatography: Ion-exchange chromatography (IEC) is used for further protein purification. In this step, proteins (including PGs) are absorbed to stationary phase with oppositely charged molecules with different absorption abilities. As the concentration gradient of eluant is gradually increased, different types of proteins can be eluted and separated [97,108]. Selection of the column packing material is essential. Diethyl amino ethyl cellulose (DEAE-cellulose) is the most commonly used for protease purification [49,50,65,67]. The buffer used as a solvent in crude enzyme extraction step is applied to equilibrate and wash the column and NaCl (with an increasing linear gradient of 0-0.5 M) and the same buffer are used to elute the PGs [65]. Tanji et al. [67] and Tanji et al. [49] used a DEAE-cellulose column eluted by a gradient of 0-0.5 M NaCl in a sodium phosphate buffer at a flow rate of 0.5 ml/min for the purification of different types of PGs from African coelacanth and North Pacific bluefin tuna. Bougateg et al. [50] used a DEAE-cellulose column equilibrated by a Tris-HCl buffer with 0-0.5M NaCl to elute the column at a flow rate of 1.3 ml/min for the purification of one type of PG from smooth hound. Wu et al. [52] and Zhou et al. [51] used a DEAE-Sephacel column and a gradient of 0-0.5 M NaCl in a sodium phosphate buffer to separate different PGs from European eel and Sea bream. The elution proceeds until no further peaks are detected, which means all proteins have been

Gel Type	Molecular Range (KDa)	References
Sephadex G-75	3–80	Gildberg and Raa [48]; Wu et al. [52]; Zhou et al. [60]
SephadexG-100	4–150	Pavlisko et al. [73]; Noda and Murakami [70]; Bougateg et al. [50]
Sephacryl S-200	5–250	Nalinanon et al. [65]; Klomklao et al. [45]; Tanji et al. [49]; Wu et al. [52]; Zhou et al. [51]

Table 3: Molecular range for gel filtration matrix in fish PG purification.

released [108]. The flow rate must be carefully controlled to encourage the elution of one type of protein at a time [48,50].

Gel filtration chromatography: Gel filtration chromatography (GFC) is used for further proteins purification based on different sizes of molecules [97,100]. Gel type is essential and each type corresponds to a distinct molecular size range. Sephadex G-75, Sephadex G-100 and Sephacryl S-200 can be used as gel matrices in the separation of different PGs (Table 3). As with ion-exchange chromatography, low flow rate gives a good resolution in gel filtration [109]. The flow rate is controlled within 10 ml/h, 20 ml/h, and 30 ml/h for the gel media Sephacryl S-200 HR, Sephadex G-50 and Sephadex G-75, respectively. Long column length and small bead size will help to achieve good resolution but the capital cost is high and the process cannot be commercialized.

GFC has been successfully used for the purification of fish pepsinogens. El-Beltagy et al. [2] used a Sephadex G-100 gel filtration column with an acetate buffer at a flow rate of 20 ml/h to collect active PG fraction from boliti fish. Wu et al. [52] used Sephacryl S-200 HR and Sephadex G-75 columns with 25 mM phosphate buffer (at flow rates of 0.6 and 0.4 ml/min, respectively) to collect pure PGs from European eel. Nalinanon et al. [65] used Sephacryl S-200 HR and Sephadex G-50 columns with a sodium phosphate buffer at a flow rate of 0.5 ml/min to collect pure PG from albacore tuna.

Aqueous two phase system (ATPS) purification of pepsinogen

The ATPS method is applied for purification of fish pepsinogen. The formation of ATPS is based on two immiscible aqueous solutions, with a polymer and a salt dispersed in two phases individually [110-112]. During the partition process, enzymes will be transferred in a large proportion towards the polymer-rich phase in the whole system. This occurs because of the strong protein-polymer interaction due to the highly hydrophobic groups of the protein surface exposed to the solvent [113]. The impurities (including soluble cells, blood, polysaccharides and pigments) are more hydrophilic and stay in the salt phase [110]. In this way, most of the enzymes can be isolated from other non-enzyme materials [111]. ATPS can yield an excellent environment which maintains the native structure of enzymes; it also allows purification by means of selective partitioning of the enzyme in one of the phases [110,113,114]. It is effective and easy to use for purification of proteins. The reagents used in this system can also be recycled, therefore reducing the cost [111]. ATPS is recognized as a promising and versatile technique for downstream processing of proteins [115].

Several researchers used ATPS for purification of proteases such as trypsin [112,116] á-chymotrypsin [117] and chymosin [111]. Klomklao et al. [116] used ATPS to purify trypsin from yellow fin tuna

and found 15% PEG1000 and 20% MgSO₄ as the best conditions for trypsin recovery (69% yield). Rawdkuen et al. [118] purified proteases from *Calotropisprocera* latex and found that 18% PEG 1000 and 14% MgSO₄ gavethe highest yield (75%). However, only a few reports were found for pepsin recovery. Nalinanon et al. [110] prepared ATPS using several different polymer-salt combinations and found the optimum to be 25% PEG 1000–20% MgSO₄ for recovering pepsinogen from albacore tuna (a yield of 85.7%). According to Nalinanon et al. [110], the storage properties of protease may change after the ATPS recovery (pepsinogen may not be affected but pepsin is subjected to reduced stability). Therefore, further studies on the separation of protease from PEG and the storage stability after this step are needed.

Activation of pepsinogen to pepsin

In this step, purified PGs are suspended in acidic condition to completely convert them to pepsins by autolysis. The pH is adjusted to 2-3 by adding 0.1M HCl at a warm temperature (25–35 °C) to maintain good enzymatic activity [51]. Under such conditions the purified PGs are activated to pepsins. The mechanism of activation of pepsinogen to pepsin involves a series of complicated reactions. At its N-terminus, the zymogen pepsinogen contains a prosegment, an additional 44 amino acid sequence, which stabilizes the inactive form and prevents the combination of substrate and active site [119]. Under acidic pHs (below 5), the electrostatic interactions and peptide bonds between the prosegment and the active enzyme are disrupted and the prosegment conformation is transformed, leading to the ultimate removal of the prosegments from the active center and, therefore, forming the active form pepsin [65,119,120]. The newly created pepsin can proteolytically cleave the prosegment from additional pepsinogen, therefore creating more pepsin [120]. Depending on the type of pepsinogen, the activation of pepsinogen proceeds in a direct pathway or a sequential pathway. In the direct pathway, pepsinogen is converted to pepsin directly, such as with PG III from North Pacific blue fin tuna [67] and PG from smooth hound [50]. In the sequential pathway, an intermediate form is involved, such as with PG from albacore tuna [65] PG II from North Pacific blue fin tuna [67] PG-I from sea bream [51] PG I and PG II from African coelacanth [49] PG-I and PG-II from Mandarin fish [60] and PG-I and PG-III from European eel [52]. The activation process lasts for 30-60 min after which the pure pepsin is obtained [65].

Advantages and disadvantages of conventional and ATPS methods

High homogeneity and purity of pepsin can be obtained by the conventional method [49,50,52]. Proper selection of chromatography can give a purification factor as high as 600 [65]. However, the conventional method usually has a low yield as more enzymes are lost during purification process. Also, the conventional methods are more complicated, time-consuming and expensive.

The ATPS method has several advantages for pepsin recovery: (a) ATPS is readily and quick to prepare (b) pepsin can be obtained by low speed centrifugation (c) it yields an excellent environment for maintaining the native structure and stability of the enzymes, (d) due to existence in different aqueous phases, the polymers and salt can be readily recycled, (e) the scale-up process based on aqueous two phase systems are simple and (f) a continuous steady state is possible [65,111,113,114]. These merits as well as continuous operation, low capital cost, eco-friendly and good feasibility give ATPS great potential for industrial application. This method is recognized as a promising and versatile technique for downstream processing of proteins [115].

The ATPS method cannot achieve as high purity as the conventional method; usually give a purification factor of less than 10. This method usually regarded as a primary purification method and other purification procedure should follow if higher purification factor is required [65].

Assaying of Pepsin

Protease assays include determination of: (a) enzyme activity, (b) enzyme concentration, (c) specific activity, (d) purification factor, (e) molecular weight, (f) homogeneity and (f) iso electric point (pI).

Enzyme activity

The most widely used method for pepsin activity (both crude and purified) was described by Anson and Mirsky (1932) and used by several researchers [2,26,33,50,51,58,59,64]. The principle is that pepsin proteolyzes acid-denatured hemoglobin (substrate) into the trichloro ethanoic acid (TCA) soluble hydrolysis products, mainly tyrosine and phenylalanine [121]. A buffer (such as glycine-HCl and sodium acetate-HCl with a pH of 2.0-3.0) is added to the substrate-enzyme mixture and incubated for 10 min at 37°C. TCA is added to terminate the enzymatic reaction and precipitate unhydrolyzed hemoglobins (incubation time of 5 min at 37°C). Centrifugation (6000 g-10,000 g for 10 min) and filtration are used to separate the supernatant from the hydrolysis products. Absorbance of the clear filtrate is measured at 280 nm directly or at 578 nm after being complexed with Folin & Ciocalteu's reagent. A blank is conducted in a similar way except that pepsin is added after the addition of TCA. Triplicate experiments are recommended for each sample determination [33].

Two types of units are used to determine total enzyme activity. One unit is defined as the amount of enzyme that produces an increase in absorbance (at 280 nm) of 0.001 per minute at a temperature of 37°C and a pH of 2-3 [33]. Another unit is expressed as the amount of enzyme that liberates 1 μmol tyrosine at a temperature of 37°C and a pH of 2-3, (absorbance at 578 nm) developed by the hydrolysis products in complexion with the Folin & Ciocalteu's reagent [122]. A standard curve of tyrosine is employed and tyrosine solutions with different concentrations ranging from 0-0.8 μmol are prepared by diluting stock tyrosine solution (1 mmol/L) with distilled water. A fitted equation of the absorbance against molar concentration of tyrosine is used to determine the total enzyme activity.

Enzyme concentration

Protein concentrations, as a proxy of pepsin concentrations, can be measured with a variety of methods that determined protein, among which Lowry and Bradford methods are most widely used. The method described by Lowry et al. [123] is based on folin-phenol-protein complexion using bovine serum albumin (BSA) as a standard. Under alkaline condition, folin-phenol (phosphor-molybdic-phosphotungstic) binds with proteins, resulting in a color change that increases the absorbance at 750 nm. A standard curve is necessary and BSA is normally used as the standard. BSA solutions with different concentrations ranging from 0-600 μg/ml are prepared by diluting a stock BSA solution (1 mg/ml) with distilled water. A fitted equation of the absorbance against BSA concentration is used to determine the total enzyme concentration. A pepsin standard curve should be employed [2,48,52,63,69,73,82]. El-Beltagy et al. [2] used Lowry method to determine the enzyme concentration and extracted 216 mg pepsin from Bolti fish. Wang et al. [82] used Lowry method and extract 26.3 mg pepsin from turbot. Xuet al. [69] used Lowry method and extracted

22.2 mg of pepsin I and 10.9 mg of pepsin II from orange roughy. Amiza and OwusuApenten [63] used Lowry method and extracted 0.057 mg purified pepsin from Atlantic cod. Noda and Murakami [70] used Lowry method and extracted 7.79 mg of pepsin I and 7.2 mg of pepsin II from sardine.

The method described by Bradford [124] is similar to the Lowry method except that it is based on the absorbance increase caused by the binding of Coomassie blue G-250 dye to proteins [125]. Standard curves are prepared in a similar way as in Lowry method except for substituting folin-phenol (dye) with Coomassie blue G-250 and would be most appropriately based on pepsin. Bradford method is faster, simpler and more sensitive than Lowry method [125]. However, it suffers from a higher detection limit. Bougateg et al. [50] used Bradford method and extracted 9.16 mg of PG from smooth hound stomach after a series of chromatography steps. Castillo-Yáñez et al. [71] used Bradford method and extracted 0.1 mg/ml of pepsin from Monterey sardine.

Specific activity (SA)

The specific activity (SA) of recovered enzymes indicates the amount of enzyme activity per unit mass of protein and is an important measurement of enzyme quality and homogeneity [126]. Pure enzyme has a constant SA under the specific substrate concentration which is defined as follows [110]:

$$SA = \frac{\text{Protein activity}}{\text{Protein concentration}} \text{ (units/mg protein)} \quad (1)$$

The specific activity is affected by the purification factor, type of fish and type of enzyme [66]. As the purity of recovered enzymes increases, the specific activity is enhanced. Usually, more purifications yield higher specific activities. Bougateg et al. [50] obtained one type of PG from smooth hound and found its specific activity increased from 0.81 U/mg protein (crude enzyme) to 7.68 U/mg protein after the purifications. Squires et al. [61] reported specific activities of 3.4, 96.4 and 108.6 U/mg protein for pepsins I, II and III from Greenland cod, respectively. Tanji et al. [67] used ASF-GFC-IEC-GFC to purify PG I, PG II and PG III from North Pacific bluefin tuna and found that the specific activities were 18.2, 41.5 and 7.8 U/mg protein, respectively. These were higher than those of 12.6, 12.8 and 1.6 U/mg protein reported by Tanji et al. [49] for PGs from African coelacanth. Noda and Murakami [70] stated that after IEC purification pepsin I from sardine had a specific activity of 53.6 U/mg proteins, which was higher than that of 40.24 U/mg protein of pepsin II. Wu et al. [52] found the potential SA of PG I from European eel to be 16.8 U/mg protein, which was lower than those of PG II and PG III (22.5 and 27.8 U/mg protein, respectively).

Purification factor (PF)

The purification factor (PF) of enzymes describes the purity of the enzyme obtained after final purification compared with the crude extract and is defined as follows [2,110]:

$$PF = \frac{SA_p}{SA_c} \quad (2)$$

Where: SA_p is the SA of purified protease

SA_c is the SA of crude extract or acidified extract

The PF may vary due to various experimental conditions. Zhou et al. [51] purified four types of pepsinogens (PG I, PG II, PG III and PG IV)

by ASF and two chromatographic steps and found their PFs to be 9.9, 9.9, 9.9 and 9.6, respectively. Similar results were reported by Wang et al. [82], Bougateg et al. [50] and Castillo-Yáñez et al. [71]. Gildberg and Raa [48] used comparable steps and obtained much purer pepsins I and II from Arctic capelin with the PFs of 42.6 and 76.9, respectively. Xu et al. [69] used ASF and three chromatographic steps to purify pepsins I and II from the stomach of orange roughy and obtained PFs of 74.3 and 76.1, respectively. Usually, increased purifications give higher purity. However, as the protease become purer, its yield is usually lowered.

Molecular weight

The molecular weight of the purified enzymes can be evaluated by the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE uses a detergent, sodium dodecyl sulfate (SDS), to denature the proteins and provide a negative charge that is proportional to the molecular weight, allowing electrophoretic separation of different protein molecules. Therefore, molecular weights of pepsin and pepsinogen can be estimated by comparisons with a series of proteins with known molecular weights. Bougateg et al. [50] found the molecular weight of one type of PG and its corresponding pepsin from smooth hound to be 40 and 35 kDa, respectively. Tanji et al. [49] used SDS-PAGE to determine the molecular weights of three PGs from African coelacanth and reported 37.0 kDa for PG I and PG II and 39.3 kDa for PG III. Zhou et al. [60] used a similar method for molecular weight determination and obtained 36, 35, 38 and 35 kDa for four purified PGs and 31, 30, 32 and 30 kDa for the four corresponding pepsins from mandarin fish, respectively. These results were similar to those reported for European eel [52], sea bream [51] and pectoral rattail [45].

Homogeneity

Native polyacryl amide gel electrophoresis (native PAGE) is often performed for analysis of homogeneity of the enzymes, which is different from SDS-PAGE. Native PAGE separates proteins according to their mass-charge ratio while in SDS-PAGE, SDS denatures and binds to the protein which is separated primarily by mass [127]. Native PAGE is not a good measurement of molecular weight as SDS-PAGE but can determine the protein charge or subunit composition. Therefore, the analyzed enzymes (maintaining their natural conformation and homogeneity) can be determined. Klomklao et al. [45] used native PAGE and found that pepsins I and II purified from pectoral rattail exhibited single band which indicated the purified enzymes were homogeneous. El-Beltagy et al. [2] used a similar method and stated that the PAGE pattern of the pepsin eluted by column chromatography revealed only one band, which proved that the enzyme was quite homogenous. Several researchers [49,57,65,67] used this method and found the final enzymes were pure and homogenous.

Isoelectric point (pI)

The isoelectric point (pI) of fish pepsin is the pH at which the enzyme molecule or surface carries no net electrical charge. The isoelectric point of pepsin is usually evaluated by analytical electro focusing in thin layer polyacrylamide flat gel (ampholyne polyacrylamide gel plate) containing ampholyne over a pH range of 3.5-9.5, with a calibration kit containing many proteins of known isoelectric points as a reference [46,71]. After separation, the proteins are stained with coomassie brilliant blue and their pI can be evaluated by comparison to reference proteins. The isoelectric points of fish pepsins, which have been determined by several researchers by the same approach range from 4.0 to 6.5. Castillo-Yáñez et al. [71] found that the isoelectric points

for two types of pepsin isolated from Monterey sardine were 4.0 and 4.5. Gildberg and Raa [48] used an analytical electrofocusing method to analyze the pI of pepsins and reported pIs of 6.6 and 3.5 for pepsins I and II from Arctic capelin, respectively. Bjelland et al. [64] found that pIs for pepsins I and II from Atlantic cod were 6.9 and 4.2, respectively. Xu et al. [69] found the pIs for pepsins I and II (a and b) from orange roughy were 5.5, 4.35 and 4.4, respectively.

Kinetics of Fish Pepsin

Catalytic reaction

When enzymes interact with specific substrates, the catalytic reaction can be described as follows [38]:



Where:

E is the enzyme

S is the substrate

ES is the enzyme-substrate complex

P is the product produced.

k_1 is the forward rate constant for substrate binding

k_{-1} is the reverse rate constant for substrate binding

k_2 is the catalytic rate constant (containing terms related to the transition state)

This reaction can be expressed by the Michaelis-Menten equation as follows:

$$V_0 = \frac{V_{\max}[S]}{K_m + [S]} \quad (4)$$

Where:

V_0 is the initial reaction rate

[S] is the substrate concentration at this time

V_{\max} is the maximum reaction rate under a specific experimental condition

K_m is Michaelis-Menten constant and refers to the substrate concentration at 1/2 maximum reaction rate

K_m can be determined by the profile of the reaction velocity at different substrate concentrations. By modifying the Michaelis-Menten equation, a linear format called Lineweaver-Burk equation is obtained [128].

$$\frac{1}{V_0} = \frac{K_m + [S]}{V_{\max}[S]} \quad (5)$$

or

$$\frac{1}{V_0} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} \quad (6)$$

By plotting $1/[S]$ versus $1/V_0$, the slope (K_m/V_{\max}) and intercept ($1/V_{\max}$) can be obtained and K_m can be calculated. k_{cat} is the turnover number, which refers to the number of substrate molecules converted to product molecules by one enzyme molecule per unit time. k_{cat} can be obtained by the following equation:

Enzyme	Identified Species	K_m (mM)	k_{cat} (S^{-1})	Reference
Pepsin	Bolti fish	0.77	—	El-Beltagy et al.[2]
Pepsin	Polar cod	1.33	—	Arunchalam and Haard [76]
Pepsin I	Pectoral rattail	0.098	50	Klomklao et al. [45]
Pepsin II	Pectoral rattail	0.152	32	Klomklao et al. [45]
Pepsin I	European eel	0.088	23.7	Wu et al. [52]
Pepsin II	European eel	0.092	19.4	Wu et al. [52]
Pepsin III	European eel	0.07	34.4	Wu et al. [52]
Pepsin I	Sea bream	0.087	—	Zhou et al. [51]
Pepsin II	Sea bream	0.1	—	Zhou et al. [51]
Pepsin III	Sea bream	0.086	—	Zhou et al. [51]
Pepsin IV	Sea bream	0.073	—	Zhou et al. [51]
Pepsin I	Atlantic cod	0.156	18	Gildberg et al. [46]
Pepsin II	Atlantic cod	0.044	33	Gildberg et al.[46]
Pepsin I	Antarctic fish	0.074	3.32	De Luca et al. [38]
Pepsin II	Antarctic fish	0.025	0.54	De Luca et al.[38]

Table 4: K_m and k_{cat} of pepsins from different fish species.

$$k_{cat} = \frac{V_{max}}{[E]} \quad (7)$$

Where:

[E] is the enzyme concentration.

Zhou et al. [51] found the K_m values for pepsins I, II, III and IV from sea bream to be 8.7×10^{-8} , 1.0×10^{-7} , 8.6×10^{-8} and 7.3×10^{-8} M, respectively. Two pepsins from Antarctic fish were found to have lower values of K_m (0.074 mM and 0.025 mM) and k_{cat} ($3.32 S^{-1}$ and $0.54 S^{-1}$) than those ($K_m = 0.26$ mM and $k_{cat} = 72.02 S^{-1}$) from pig pepsin [38]. Klomklao et al. [45] found the K_m values of two pepsins from pectoral rat tail were 0.098 and 0.152 mM and k_{cat} values were 50 and 32 S^{-1} . Pepsin from polar cod exhibited a higher K_m than those obtained from warm water fishes [30,46,64,74,76]. A summary of K_m and k_{cat} values for different pepsins is shown in Table 4.

Partition coefficient (K_p)

The partition coefficient (K_p) of recovered proteases is used to characterize the protein distribution in ATPS and is defined as follows [110]:

$$k_p = \frac{C_T}{C_B} \quad (8)$$

Where:

C_T is the concentration of protein in top phase

C_B is the concentration of protein in bottom phase

Generally, a low partition coefficient indicates transfer of more proteins, nucleic acids and other contaminants to the bottom phase [116,118]. The phase system composition (salt type and concentration and PEG molecular weight and concentration) can influence the

partition of phase components and therefore have a significant influence on K_p [110]. Klomklao et al. [116] investigated partition coefficients during spleen proteinase isolation and found that 20% PEG1000–20% $MgSO_4$ yielded a low K_p of 0.32 (less impurity) while 20% PEG 1000–20% $(NH_4)_2SO_4$ gave a high value (1.73). Rawdkuen et al. [118] used a similar method for protease recovery from the latex of *Calotropisprocera* and found the phase combination 18% PEG 1000–20% $MgSO_4$ had the lowest K_p (0.03) while 18%PEG 1000–20% K_2HPO_4 had the highest value (4.0). However, K_p only indicates the movement of protein mixture (including target enzymes and undesirable proteins) instead of a single enzyme. Usually, good partition results from a moderate K_p (highest or lowest values cannot give good partitioning).

Volume ratio (V_R)

The Volume Ratio (V_R) of recovered proteases shows the phase volume distribution in ATPS and is defined as follows [110].

$$V_R = \frac{V_T}{V_B} \quad (9)$$

Where:

V_T is the volume of solution in the top phase

V_B is the volume of solution in the bottom phase

Nalinanon et al. [110], Chaiwut et al. [129] and Rawdkuen et al. [118] reported that the V_R decreased when salt concentration increased. Increasing salt concentration from 11% to 17% gave a higher proportion of salt-rich bottom phase, therefore, resulting in decrease in V_R [129]. Nalinanon et al. [110] investigated the pepsin partition from albacore tuna and reported that, with the same polymer (20% PEG 1000), V_R decreased from 0.91 to 0.59 as K_2HPO_4 increased from 15% to 25%. Chaiwut et al. [129] reported that when the concentration of $(NH_4)_2SO_4$ increased from 14% to 17% with the fixed polymer (12% PEG 4000), V_R was reduced from 1.17 to 0.42 during protease extraction from *Calotropisprocera* latex.

Recovery yield

The recovery yield of protease is a characteristic parameter showing recovery efficiency. It is calculated from the ratio of protease activities in ATPS and defined as follows [110]:

$$Yield(\%) = \frac{A_i}{A_j} \times 100 \quad (10)$$

Where:

A_i is enzyme activity of purified protease

A_j is the enzyme activity of crude extract or acidified extract.

High values of recovery yield indicates that more desired enzymes were partitioned from the original material and experimental conditions gave an effective recovery. Squires et al. [61] used a conventional purification method (ASF, ultrafiltration and a series of chromatographic steps) to extract pepsins from Greenland cod and found the yields of pepsins I, II and III were 4.4%, 14.3% and 13.0%, respectively. These results were similar to those for Arctic capelin reported by Gildberg and Raa [48] and for mandarin fish reported by Zhou et al. [60]. Noda and Murakami [70] purified pepsins I and II from sardine and found lower yields (3.4% and 1.9% for pepsins I and II, respectively). Similar results were reported by Wu et al. [52] and Twining et al. [57]. As the number of purification steps increased, enzyme yield will be greatly reduced.

Conclusion

Pepsin is an important aspartic proteases existing in the fish stomach. Its enzymatic activity can be affected by three factors: pH, temperature and inhibitor. Each type of fish pepsin has a distinct optimum pH, pH stability, an optimum temperature and temperature stability. Warm water fish pepsins have a higher optimum temperature and a higher upper temperature limit of 40-50°C while cold water fish pepsins are quite susceptible to higher temperatures. Pepstatin A with a specific molar pepstatin to pepsin ratio and sodium dodecyl sulfate (SDS) at 0.05–0.10% (w/v) have strong inhibitory effects on pepsin activity while PMSF, E-64 and EDTA have a negligible impact on pepsin activity. Cysteine and aliphatic alcohols, have been identified as effective inhibitors while ATP, molybdate, NaCl, MgCl₂, and CaCl₂ are not. Pepsin is used in collagen extraction, in medical research, as a rennet substitute, in the production of fish silage and in digestibility therapy. Pepsins are partition edusing a four step procedure including preparation of the fish stomach, extraction of crude PG, purification of PG and activation of PG to pepsin at an acidic pH. The purification of PG is achieved by the conventional method and aqueous two phase system (ATPS). The conventional method is used for highly purified pepsin and involves ammonium sulfate fractionation, gel filtration or anion exchange chromatography. ATPS is used for partially purified pepsin and is based on two immiscible aqueous polymer-salt solutions. The innovative methods for pepsin partition have been proposed but large scale of pepsin recovery is still in demand in the industry at present. The characteristics of fish pepsin are assayed by enzyme activity and concentration, specific activity (SA), purification factor (PF), molecular weight, homogeneity and isoelectric point (pI). Pepsin is considered a promising protease with wide applications in both conventional and new industries and new efficient, cost effective techniques for its recovery and purification should be developed.

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References

- Gildberg A, Raa J (1998) Properties of a propionic acid/formic acid preserved silage of cod viscera. J Sci Food Agric 28: 647-653.
- El-Beltagy AE, El-Adawy TA, Rahma EH, El-Bedawy AA (2004) Purification and characterization of an acidic protease from the viscera of boliti fish (*Tilapia nilotica*). Food Chem 86: 33-39.
- Nagai T, Suzuki N (2000) Isolation of collagen from fish waste material – skin, bone and fins. Food Chem 68: 277-281.
- Ogawa M, Portier RJ, Moody MW, Bell J, Schexnayder MA, et al. (2004) Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogoniscromis*) and sheepsheadseabream (*Archosargus probatocephalus*). Food Chem 88: 495-501.
- Yoshida H, Terashima M, Takahashi Y (1999) Production of organic acids and amino acids from fish meat by sub-critical water hydrolysis. Biotechnol Prog 15: 1090-1094.
- Genicot S, Rentier-Delrue F, Edward D, VanBeeumen J, Gerday C (1996) Trypsin and trypsinogen from an Antarctic fish: molecular basis of cold adaptation. Biochim Biophys Acta 1298: 45-57.
- Klomkiao S, Benjakul S, Visessanguan W, Kishimura H, Simpson BK (2006) Purification and characterization of trypsin from the spleen of tongol tuna (*Thunnustonggol*). J Agric Food Chem 54: 5617-5622.
- Castillo-Yáñez FJ, Pacheco-Aguilar R, García-Carreño FL, Navarretedel Toro MA, López MF (2006) Purification and biochemical characterization of chymotrypsin from the viscera of monterey sardine (*Sardinops sagax caeruleus*). Food Chem 99: 252-259.
- Gildberg A, Øverbø K (1990) Purification and characterization of pancreatic elastase from Atlantic cod (*Gadus morhua*). Comp Biochem Physiol B 97: 775-782.
- Wiggers VR, Wisniewski Jr A, Madureira LAS, Chivanga Barros AA, Meier HF (2009) Biofuels from waste fish oil pyrolysis: Continuous production in a pilot plant. Fuel 88: 2135-2141.
- Jongjareonrak A, Benjakul S, Visessanguan W, Nagai T, Tanaka M (2005) Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of brownstripe red snapper (*Lutjanus vitta*). Food Chem 93: 475-484.
- Zhang Y, Liu W, Li G, Shi B, Miao Y, et al. (2007) Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*). Food Chem 103: 906-912.
- Nalinanon S, Benjakul S, Visessanguan W, Kishimura H (2007) Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthustayenus*). Food Chem 104: 593-601.
- Nalinanon S, Benjakul S, Visessanguan W, Kishimura H (2008) Improvement of gelatin extraction from bigeye snapper skin using pepsin-aided process in combination with protease inhibitor. Food Hydrocolloids 22: 615-622.
- Aehle W (2007) Enzymes in industry: production and applications. (3rd edn), Wiley-VCH, Weinheim, Germany, 136-137.
- Thorne Research (2010) Betaine HCl and Pepsin; Dover, USA.
- Sogawa K, Fujii-Kuriyama Y, Mizukami Y, Ichihara Y, Takahashi T (1983) Primary structure of human pepsinogen gene. J Biol Chem 258: 5306-5311.
- Sogawa K, Fujii-Kuriyama Y, Mizukami Y, Ichihara Y, Takahashi T (1983) Primary structure of human pepsinogen gene. J Biol Chem 258: 5306-5311.
- Kageyama T, Takahashi K (1976) Pepsinogens and pepsins from gastric mucosa of Japanese monkey. Purification and characterization. J Biochem 79: 455-468.
- Nielsen PK, Foltmann B (1995) Purification and characterization of porcine pepsinogen B and pepsin B. Arch Biochem Biophys 322: 417-422.
- Martin P, Torieu-Cuot P, Collin JC, Ribadeau Dumas B (1982) Purification and characterization of bovine gastricsin. Eur J Biochem 122: 31-39.
- Suzuki M, Narita Y, Oda S, Moriyama A, Takenaka O, et al. (1999) Purification and characterization of goat pepsinogens and pepsins. Comp Biochem Physiol B Biochem Mol Biol 122: 453-460.
- Muto N, Tani S (1979) Purification and characterization of rat pepsinogens whose contents increase with developmental progress. J Biochem 85: 1143-1149.
- Kageyama T, Tanabe K, Koiwai O (1990) Structure and development of rabbit pepsinogens. Stage-specific zymogens, nucleotide sequences of cDNAs, molecular evolution, and gene expression during development. J Biol Chem 265: 17031-17038.
- Gildberg A (1992) Recovery of proteinases and protein hydrolysates from fish viscera. Bioresource Technol 39: 271-276.
- Simpson BK (2000) Digestive proteinases from marine animals. In Seafood enzymes: utilization and influence on postharvest seafood quality. Haard HF, Simpson BK, Eds. Marcel Dekker, Inc: New York, 191-213.
- Fruton JS (1971) Hydrolysis: peptide bonds, (3rd edn), Academic Press Inc: New York 3: 120-152.
- Effront J, Prescott SC, Venable CS (2007) Biochemical catalysts in life and industry: proteolytic enzymes. Kessinger Publishing: 151-289.
- Bobe J, Goetz FW (2001) An ovarian progastricsin is present in the trout coelomic fluid after ovulation. Biol Reprod 64: 1048-1055.
- Kurokawa T, Uji S, Suzuki T (2005) Identification of pepsinogen gene in the genome of stomachless fish, *Takifugu rubripes*. Comp Biochem Physiol B Biochem Mol Biol 40: 133-140.

30. Shahidi F, Janak Kamil YVA (2001) Enzymes from fish and aquatic invertebrates and their application in the food industry. Trends Food Sci Technol 12: 435-464.
31. Raufman J (2004) Pepsin. In Encyclopedia of Gastroenterology, Johnson L R. Ed., Academic Press, Amsterdam 3: 147-148.
32. Karlsen S, Hough E, Olsen RL (1998) Structure and proposed amino-acid sequence of a pepsin from Atlantic cod (*Gadus morhua*). Acta Crystallogr D Biol Crystallogr 54: 32-46.
33. Andreeva NS, Rumsh LD (2001) Analysis of crystal structures of aspartic proteinases: On the role of amino acid residues adjacent to the catalytic site of pepsin-like enzymes. Protein Sci 10: 2439-2450.
34. Worthington V (2010) Worthington enzyme manual: enzymes and related biochemicals; Worthington Biochemical Corporation, U.S.A.
35. Suguna K, Padlan EA, Smith KW, Carlson WD, Davies DR (1987) Binding of a reduced peptide inhibitor to the aspartic proteinase from *Rhizopus chinensis*: Implications for a mechanism of action. Proc Natl Acad Sci U S A 84: 7009-7013.
36. Davies DR (1990) The Structure and Function of the Aspartic Proteinases. Annu Rev Biophys Chem 19: 189-215.
37. Benjakul S, Klomkiao S, Simpson BK (2010) Enzymes in fish processing. In Enzymes in Food Technology (2nd edn), Whitehurst RJ, Van Oort M, Eds. Blackwell Publishing, Chichester: 211-225.
38. De Luca V, Maria G, De Mauro G, Catara G, Carginale V, et al. (2009) Aspartic proteinases in Antarctic fish. Mar Genomics 2: 1-10.
39. Polgár L (1987) The mechanism of action of aspartic proteases involves "push-pull" catalysis. FEBS Lett 219: 1-4.
40. Deng Y, Zhao J, Lu G, Wu X, Tao Y (2010) Cloning, characterization and expression of the pepsinogen C from the golden mandarin fish *Siniperca scherzeri* (Teleostei: Perciformes). Fish Sci 76: 819-826.
41. Foltmann B (1981) Gastric proteinases-structure, function, evolution and mechanism of action. Essays Biochem 17: 52-84.
42. Cooper JB, Khan G, Taylor G, Tickle IJ, Blundell TL (1990) X-ray analyses of aspartic proteinases. II. Three-dimensional structure of the hexagonal crystal form of porcine pepsin at 2.3 Å resolution. J Mol Biol 214: 199-222.
43. Kageyama T (2002) Pepsinogen, progastricsin, and prochymosin: structure, function, evolution, and development. Cell Mol Life Sci 59: 288-306.
44. Tanji M, Yakabe E, Kubota K, Kageyama T, Ichinose M, et al. (2009) Structural and phylogenetic comparison of three pepsinogens from Pacific bluefin tuna: molecular evolution of fish pepsinogens. Comp Biochem Physiol B Biochem Mol Biol 152: 9-19.
45. Klomkiao S, Kishimura H, Yabe M, Benjakul S (2007) Purification and characterization of two pepsins from the stomach of pectoral rattail (*Coryphaenoides spectoralis*). Comp Biochem Physiol B Biochem Mol Biol 147: 682-689.
46. Gildberg A, Olsen RL, Bjarnason JB (1990) Catalytic properties and chemical composition of pepsins from Atlantic cod (*Gadus morhua*). Comp Biochem Physiol B 96: 323-330.
47. Feng S, Li W, Lin H (2008) Characterization and expression of the pepsinogen C gene and determination of pepsin-like enzyme activity from orange-spotted grouper (*Epinephelus coioides*). Comp Biochem Physiol B 149: 275-284.
48. Gildberg A, Raa J (1983) Purification and characterization of pepsins from the arctic fish capelin (*Mallotus villosus*). Comp Biochem Physiol A Comp Physiol 75: 337-342.
49. Tanji M, Yakabe E, Kageyama T, Yokobori S, Ichinose M, et al. (2007) Purification and characterization of pepsinogens from the gastric mucosa of African coelacanth, *Latimeria chalumnae*, and properties of the major pepsins. Comp Biochem Physiol B Biochem Mol Biol 146: 412-420.
50. Bougateg A, Balti R, Zaied SB, Souissi N, Nasri M (2008) Pepsinogen and pepsin from the stomach of smooth hound (*Mustelus mustelus*): Purification, characterization and amino acid terminal sequences. Food Chem 107: 777-784.
51. Zhou Q, Fu X, Zhang L, Su W, Cao M Purification and characterization of sea bream (*Sparus latus Houlttuyn*) pepsinogens and pepsins. Food Chem 103: 795-801.
52. Wu T, Sun L, Du C, Cai Q, Zhang Q, et al. (2009) Identification of pepsinogens and pepsins from the stomach of European eel (*Anguilla anguilla*). Food Chem 115: 137-142.
53. Gildberg A (2004) Enzymes and bioactive peptides from fish waste related to fish silage, fish feed and fish sauce production. J Aquat Food Prod Technol 13: 3-11.
54. Haard NF (1992) Control of chemical composition and food quality attributes of cultured fish. Food Res Int 25: 289-307.
55. García-Carreño FL, Hernández-Cortés MP, Haard NF (1994) Enzymes with peptidase and proteinase activity from the digestive systems of a freshwater and a marine decapod. J Agric Food Chem 42: 1456-1461.
56. Gildberg A (1998) Aspartic proteinases in fishes and aquatic invertebrates. Comp Biochem Physiol B 91: 425-435.
57. Twining SS, Alexander PA, Huijbregtse K, Glick DM (1983) A pepsinogen from rainbow trout. Comp Biochem Physiol B 75: 109-112.
58. Gildberg A (2004) Digestive enzyme activities in starved pre-slaughter farmed and wild-captured, Atlantic cod (*Gadus morhua*). Aquaculture 238: 343-353.
59. Brier S, Maria G, Carginale V, Capasso A, Wu Y, et al. (2007) Purification and characterization of pepsins A1 and A2 from the Antarctic rock cod *Trematomus bernacchii*. FEBS J 274: 6152-6166.
60. Zhou Q, Liu G, Huang Y, Weng L, Hara K, et al. (2008) Pepsinogens and pepsins from mandarin fish (*Siniperca chuatsi*). J Agric Food Chem 56: 5401-5406.
61. Squires EJ, Haard NF, Feltham LA (1986) Gastric proteases of the Greenland cod *Gadus ogac*. I. Isolation and kinetic properties. Biochem Cell Biol 64: 205-214.
62. Haard NF (1986) Atlantic Cod Gastric Protease. Characterization with casein and milk substrate and influence of sepharose immobilization on salt activation, temperature characteristics and milk clotting reaction. J Food Sci 51: 313-316.
63. Amiza MA, Owusu Apenten RK (2002) A single step purification of gastricsin-like proteinase from Atlantic cod (*Gadus morhua*). OnLine J Biol Sci 2: 591-595.
64. Bjelland S, Gildberg A, Volden G (1988) Degradation of human epidermal keratin by fish pepsin. Arch Dermatol Res 280: 119-123.
65. Nalinanon S, Benjakul S, Kishimura H (2010) Biochemical properties of pepsinogen and pepsin from the stomach of albacore tuna (*Thunnus alalunga*). Food Chem 121: 49-55.
66. Nalinanon S, Benjakul S, Visessanguan W, Kishimura H (2008) Tuna pepsin: characteristics and its use for collagen extraction from the skin of threadfin bream (*Nemipterus spp.*). J Food Sci 73: C413-419.
67. Tanji M, Kageyama T, Takahashi K (1988) Tuna pepsinogens and pepsins. Purification, characterization and amino-terminal sequence. Eur J Biochem 177: 251-259.
68. Norris ER, Mathies JC (1953) Preparation, properties, and crystallization of tuna pepsin. J Biol Chem 204: 673-680.
69. Xu RA, Wong RJ, Rogers ML, Fletcher GC (1996) Purification and characterization of acidic proteases from the stomach of the deepwater finfish orange roughy (*Hoplostethus atlanticus*). J Food Biochem 20: 31-48.
70. Noda M, Murakami K (1981) Studies on proteinases from the digestive organs of sardine. II. purification and characterization of two acid proteinases from the stomach. Biochim Biophys Acta 658: 27-34.
71. Castillo-Yáñez FJ, Pacheco-Aguilar R, García-Carreño FL, Navarrete-del Toro MA (2004) Characterization of acidic proteolytic enzymes from monterey sardine (*sardinops sagax caerulea*) viscera. Food Chem 85: 343-350.
72. Haard NF, Shamsuzzaman K, Brewer P, Arnchalam K (1982) Enzymes from marine organisms as rennet substitutes. In Use of enzymes in food technology. Symposium International Versailles, Dupuy P, Ed., Technique et Documentation Lavoisier, Paris, 237-241.

73. Pavlisko A, Rial A, Devecchi S, Coppes Z (1997) Properties of pepsin and trypsin isolated from the digestive tract of *Parona signata* "Palometa". J Food Biochem 21: 289-308.
74. Simpson BK, Haard NF (1987) Cold-adapted enzymes from fish. In Food Biotechnology, Knorr DW, Ed, Marcel Dekker Inc, New York, 495-527.
75. Guerard F, Le Gal Y (1987) Characterization of a chymosin-like pepsin from the dogfish *Scyliorhinus canicula*. Comp Biochem Physiol B 88: 823-827.
76. Arunchalam K, Haard NF (1985) Isolation and characterization of pepsin from polar cod (*Boreogadus saida*). Comp Biochem Physiol B 80: 467-473.
77. Copeland RA (2005) Evaluation of enzyme inhibitors in drug discovery: A guide for medicinal chemists and pharmacologists. John Wiley and Sons Inc, Hoboken, New Jersey, 34-66.
78. Umezawa H, Aoyagi T, Morishima H, Matsuzaki M, Hamada M (1970) Pepstatin, a new pepsin inhibitor produced by *Agmatomyces*. J Antibiot (Tokyo) 23: 259-262.
79. Zollner H (1999) Handbook of enzyme inhibitors. (3th edn), Wiley-VCH, Chichester, UK, 440-445.
80. Yakabe E, Tanji M, Ichinose M, Goto S, Miki K, et al. (1991) Purification, characterization, and amino acid sequences of pepsinogens and pepsins from the esophageal mucosa of bullfrog (*Rana catesbeiana*). J Biol Chem 266: 22436-22443.
81. Tang J (1965) Competitive inhibition of pepsin by aliphatic alcohols. J Biol Chem 240: 3810-3815.
82. Wang H, Wang Y, Wang Q, Xue C, Sun M (2006) Purification and characterization of stomach protease from the turbot (*Scophthalmus maximus* L.) Fish Physiol Biochem 32: 179-188.
83. Nagai T, Yamashita E, Taniguchi K, Kanamori N, Suzuki N (2001) Isolation and characterization of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*). Food Chem 72: 425-429.
84. Ahmad M, Benjakul S (2010) Extraction and characterisation of pepsin-solubilised collagen from the skin of unicorn leatherjacket (*Aluterus monoceros*). Food Chem 120: 817-824.
85. Gorgas FJS (2009) Dental medicine. A manual of dental material medica and therapeutics, Nabu Press, Washington, 48-50.
86. Murado MA, González MDP, Vázquez JA (2009) Recovery of proteolytic and collagenolytic activities from viscera by-products of rayfish (*Raja clavata*). Mar Drugs 7: 803-815.
87. Almás KA (1990) Utilization of marine biomass for production of microbial growth media and biochemicals. In Advances in Fisheries Technology and Biotechnology for increased profitability, Voigt MN, Botta JR, Eds., Technomic Publishing Company, Lancaster, 361-372.
88. Brewer P, Helbig N, Haard NF (1984) Atlantic cod pepsin. Characterization and use as a rennet substitute. Can Inst Food Sci Technol 17: 38-43.
89. Tavares JFP, Baptista JAB, Marcone MF (1997) Milk-coagulating enzymes of tuna fish waste as a rennet substitute. Int J Food Sci Nutr 48: 169-176.
90. Raa J, Gildberg A (1982) Fish silage: A review. Crit Rev Food Sci Nutr 16: 383-419.
91. Gildberg A, Almás KA (1986) Utilization of fish viscera. In Food Engineering and Process Application, LeMaguer M, Jelen P, Eds, Elsevier Applied Science Publishers, London, 383-393.
92. Raa J, Gildberg A (1976) Autolysis and proteolytic activity of cod viscera. Int J Food Sci Technol 11: 619-628.
93. Windsor M, Barlow S (1981) Introduction to fishery by-products. Fishing News Books, Farnham, UK, 153-154.
94. Goddard JS, Al-Yahyai DSS (2001) Chemical and nutritional characteristics of dried sardine silage. J Aquat Food Prod Technol 10: 39-50.
95. Saisithi P (1994) Traditional fermented fish: Fish sauce production. In Fisheries Processing: Biotechnological Applications, Martin AM, Eds, Chapman and Hall, London, 111-131.
96. Joakimsson KG (1984) Enzymatic deskinning of herring (*Clupea harengus*). Ph.D. thesis, Institute of Fisheries, University of Tromsø, Tromsø, Norway, 54.
97. Roe SD (2001) Purification strategy. In Protein purification techniques (2nd edn), Roe S, Eds, Oxford University Press Inc, New York, 1-10.
98. England S, Seifter S (1990) Precipitation techniques. Methods in Enzymology Guide to Protein Purification, Deutscher MP, Eds, Academic Press Inc, San Diego, California, 182: 285-300.
99. Rossomando EF (1990) Ion-exchange chromatography. In Methods in Enzymology. Guide to Protein Purification, Deutscher MP, Eds, Academic Press Inc, San Diego, California 182: 309-316.
100. Stellwagen E (1990) Gel filtration. In Methods in Enzymology. Guide to Protein Purification, Deutscher MP, Ed, Academic Press Inc, San Diego, California 182: 317-328.
101. Bianchi M, Boigegrain RA, Castro B, Coletti-Previero M (1990) N-terminal domain of pepsin as a model for retroviral dimeric aspartyl protease. Biochem Biophys Res Commun 167: 339-344.
102. Tanji M, Yakabe E, Kageyama T, Takahashi K (1996) The primary structure of the major pepsinogen from the gastric mucosa of tuna stomach. J Biochem 120: 647-656.
103. Doonan S (1996) Preparation of extracts from animal tissues. In Protein purification protocols, Doonan S, Ed, Humana Press Inc, Totowa, New Jersey, 17-22.
104. Chong ASC, Hashim R, Chow-Yang L, Ali AB (2002) Partial characterization and activities of proteases from the digestive tract of discus fish (*Symphysodon aequifasciata*). Aquaculture 203: 321-333.
105. Chiu S, Pan BS (2002) Digestive protease activities of juvenile and adult eel (*Anguilla japonica*) fed with floating feed. Aquaculture 205: 141-156.
106. Ersson B, Rydén L, Janson J (1998) Introduction to protein purification. In Protein purification: principles, high-resolution methods, and applications, (2nd edn), Janson J, Rydén L, Eds, John Wiley, Sons Inc, New York, 3-40.
107. Doonan S (2004) Bulk purification by fractional precipitation. In Protein purification protocols, Cutler P, Eds, Humana Press Inc, Totowa, New Jersey, 117-124.
108. Yamamoto S, Nakanishi K, Matsuno R (1988) Ion-exchange chromatography of proteins, M. Dekker, New York, 13-16.
109. Marshak DR, Kadonaga JT, Burgess RR, Knuth MW, Brennan WA (1996) Strategies for Protein Purification and Characterization: A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, New York, 127-201.
110. Nalinanon S, Benjakul S, Visessanguan W, Kishimura H (2009) Partitioning of protease from stomach of albacore tuna (*Thunnus alalunga*) by aqueous two-phase systems. Process Biochem 44: 471-476.
111. Spelzini D, Farruggia B, Picó G (2005) Features of the acid protease partition in aqueous two-phase systems of polyethylene glycol-phosphate: chymosin and pepsin. J Chromatogr B 821: 60-66.
112. Tubío G, Nerli B, Picó G (2007) Partitioning features of bovine trypsin and α -chymotrypsin in polyethyleneglycol-sodium citrate aqueous two phase system. J Chromatogr B 852: 244-249.
113. Imelio N, Marini A, Spelzini D, Picó G, Farruggia B (2008) Pepsin extraction from bovine stomach using aqueous two-phase systems: Molecular mechanism and influence of homogenate mass and phase volume ratio. J Chromatogr B 873: 133-138.
114. Tanuja S, Srinivas ND, RaghavaRao KSMS, Gowthaman MK (1997) Aqueous two-phase extraction for downstream processing of amyloglucosidase. Process Biochem 32: 635-641.
115. Albertsson P, Cajarville A, Brooks DE, Tjerneld F (1987) Partition of proteins in aqueous polymer two-phase systems and the effect of molecular weight of the polymer. Biochim Biophys Acta 926: 87-93.
116. Klomkloa S, Benjakul S, Visessanguan W, Simpson BK, Kishimura H (2005) Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems. Process Biochem 40: 3061-3067.

117. Oliveira LA, Sarubbo LA, Porto ALF, Campos-Takaki GM, Tambourgi EB (2002) Partition of trypsin in aqueous two phase systems of polyethylene glycol and cashew-nut tree gum. *Process Biochem* 38: 693-699.
118. Rawdkuen S, Pintathong P, Chaiwut P, Benjakul S (2011) The partitioning of protease from *Calotropis procera* latex by aqueous two-phase systems and its hydrolytic pattern on muscle proteins. *Food and Bioprocess* 89: 73-80.
119. Richter C, Tanaka T, Yada RY (1998) Mechanism of activation of the gastric aspartic proteinases: pepsinogen, progastricsin and prochymosin. *Biochem J* 335: 481-490.
120. Sanny CG, Hartsuck JA, Tang J (1975) Conversion of pepsinogen to pepsin. Further evidence for intramolecular and pepsin-catalyzed activation. *J Biol Chem* 250: 2635-2639.
121. Fourie J, Arnot RS, Carter J, Hickman R, Terblanche J (1974) Measurement of pepsin in porcine gastric juice. *S Afr Med J* 48: 1873-1875.
122. Northrop JH (1932) Pepsin activity units and methods for determining peptic activity. *J Gen Physiol* 16: 41-58.
123. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. *J Biol Chem* 193: 256-275.
124. Bradford MA (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72: 248-254.
125. Nigam A, Ayyagari A (2007) *Lab manual in biochemistry, immunology and biotechnology*; Tata McGraw-Hill Publishing Company, New Delhi, 53-54.
126. Passonneau JV, Lowry O H (1993) *Enzymatic analysis: A practical guide*, The Humana Press Inc, Totowa, New Jersey, 229-306.
127. Speicher DW (2005) Electrophoresis. In *Current protocols in protein science*, Coligan, JE, Eds, John Wiley and Sons Inc, New York, 36-35.
128. Lineweaver H, Burk D (1934) The determination of enzyme dissociation constants. *J Am Chem Soc* 56: 658-666.
129. Chaiwut P, Rawdkuen S, Benjakul S (2010) Extraction of protease from *calotropis procera* latex by polyethylene glycol-salts biphasic system. *Process Biochem* 45: 1148-1155.