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Sustainable exploitation of fish visceral proteases: Molecular characteristics and diverse applications

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ABSTRACT: Proteases, a class of hydrolases, are widely used in several industries. Fish proteolytic enzymes can be a potential alternative since they are mainly from viscera, the leftover from fish/shellfish processing industry. As a consequence, the viscera can be utilized more effectively, instead of being used as animal feed, fertilizer, or being discarded. These viscera contribute to environmental problems due to the rapid autolysis associated with microbial spoilage. Pepsin and trypsin, the primary digestive enzymes found in fish viscera, exhibit high hydrolytic activity toward various proteins under their optimal condition. Additionally, several fish enzymes have a cold-adapted nature, distinct from those of other origins. Their high catalytic activity at low temperature makes them well-suited for diverse applications in food processing aid, meat tenderization, etc., without compromising the quality and acceptability. However, partial purification is required to concentrate the proteases and remove the undesirable impurities, especially fat or offensive odorous compounds. This review describes the molecular characteristics of visceral proteases with the focus on trypsin and pepsin, the predominant proteases in the digestive organs of fish and shellfish. Various applications of fish visceral proteases include (1) assisting the extraction of pepsin soluble collagen from fish skin or scale and carotenoids from shrimp leftover, (2) production of protein hydrolysates or active peptides with bioactivities, (3) manufacturing of virgin coconut oil, and (4) other applications such as cheese making, fish silage production, extraction of carotenoprotein, and meat tenderization. Their use as a digestive aid for patients suffering from maldigestion was also revisited.

KEYWORDS: pepsin, trypsin, visceral proteases, fish/shellfish, digestive organs, industrial enzymes

INTRODUCTION

There is a considerable need for enzymes possessing particular combinations of properties tailored for various industrial applications. In 2023, global sales of industrial enzymes were estimated to be around $6.1 billion. This market is projected to grow at a compound annual growth rate (CAGR) of approximately 4.4% from 2023 to 2030, reaching nearly $8.2 billion (https://www.researchandmarkets.com/report/enzymes). Proteases play a crucial role in the digestion and breakdown of proteins, which enhances the bioavailability of nutrients necessary for growth and metabolism. Although several enzymes including lipases, amylases, and nucleases also play important roles in digestion, proteases have been specifically highlighted due to their abundance and predominant activity in the visceral organs. In industry, proteases are predominantly obtained from animals, plants, and microbiological sources. However, proteases from aquatic and other marine sources have not been applied extensively. Despite the diverse range of enzymes available from the microbial world, only a small number of microorganism species are currently employed for industrial enzyme production. This limitation is largely due to rigorous toxicological issues, in which microbes used as sources of proteases must be proven for safety. This screening process is both expensive and tedious [1]. Moreover, plant and animal proteases are traditionally preferred as food processing aids over microbial counterparts due to consumer concerns about products derived from microorganisms. However, religious belief restrict usage of enzymes from bovine and porcine origins [2]. The aquatic environment has diverse species adapted to various habitat situations. These organisms comprise finfish (Teleosti), which consist of approximately 7,000 species in freshwater and 13,000 species in saltwater environments [1]. Genetic differences among various species, coupled with variation to diverse conditions in the environment, have led to variation in the properties of proteases from fish, compared to those derived from other sources [3]. Certain enzymes sourced from aquatic organisms are presently employed as processing aid in the seafood industry [4], and this is anticipated to expand further with the current advancements in recombinant DNA technology, which facilitates the cloning of protease gene into 'generally recognized as safe' (GRAS) organisms [5]. Overall, fish viscera, discarded from fish/shellfish processing, is another crucial source of proteases. Recovery of proteases from viscera can fit well with Bio-Green-Circular (BCG) economy model.

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In the modern world, human life is highly dependent on aquatic resources as the potential food rich in nutrients, especially proteins and oil. In general, fish is directly consumed as food, and the processing leftovers are converted into fish meal and oil, mainly as the ingredients for animal feed formulation. Those inedible parts such as head, viscera, frames, and bones are also discarded without proper management, thus causing pollution worldwide. Hence, there is a strong need to utilize these wastes to produce beneficial or high-value products by prioritizing their demand in the market. Initially, Strøm and Eggum [6] documented that fish heads and internal organs were converted into fish flour powder that was fed to animals. These leftovers could be converted into fish protein hydrolysate (FPH) [4]. FPH has several potential uses such as nutritious components in animal feed [7] or functional food due to their bioactivities [2]. FPH can be added to microbial growth media as a peptone substitute [8] or as fertilizer for several plants [9].

Fish viscera refer to the internal organs of fish such as the liver, intestines, pyloric caeca, spleen and stomach, etc., which are rich in various enzymes, including proteases. Proteases obtained from fish viscera have effective applications in the food industry, pharmaceuticals or laundry industries, etc. The extraction and processing of proteases from fish viscera have been recommended over other land animal proteases because of its low cost. Fish proteases have no constraints, regardless of religions or belief. Furthermore, the unique characteristic feature of proteases from fish viscera, particularly high catalytic efficiency at lower temperature, has drawn attention for the users due to the ease of operation with low energy consumption. Recovery of proteases from fish/shellfish processing wastes and their application in industries would contribute significantly to reduction of pollution problem [10]. Complete usage of entire fish is also necessary, not only for the production of meat but also for the exploitation of viscera, e.g., utilization of visceral proteases [11]. Better exploitation of the catch would reduce over-fishing, while providing new products in the market, thus increasing income for the fish processing business. Hence, this review aims to provide an overview of molecular characteristics of proteases from viscera, and their potential uses in the food and related industries are also revisited.

FISH PROTEASES: STRUCTURES, SOURCES, AND CLASSIFICATION

Fish viscera contain various types of proteases, including both acidic and alkaline proteases. Fish and aquatic invertebrate proteases fall into four main categories according to the International Union of Applied Biochemists' categorization system: Aspartic (carboxyl) proteases, serine proteases, cysteine (thiolic) proteases, and metalloproteases [12]. Among the digestive proteases, pepsin and trypsin are extensively preferred to be recovered due to its high efficacy and potential applications in the industries.

Pepsin

Pepsin is an aspartic protease, which can digest proteins in the stomach at an acidic pH range. Aspartic proteases are a type of endopeptidases recognized for their excellent stability and activity at acidic pH [13]. Usually, aspartic proteases are mentioned as "aspartyl" proteases or carboxyl proteinases as their catalytic sites consist of two aspartic acid residues with a carboxyl group attached [13]. According to the enzyme commission system (EC), all the aspartic proteases of marine origin are labeled with common first 3 digits EC 3.4.23. In addition, three usual aspartic proteases purified and characterized from the aquatic animal stomach include gastricsin, chymosin, and pepsin [12].

Pepsinogen (PG), with a molecular weight (MW) of 40 kDa, is an inactive form of pepsin that is produced and secreted in the stomach membrane. In comparison with pepsin, PG has an extra 44 amino acids and is stable in weak alkaline and neutral conditions. Those 44 amino acids are proteolytically eliminated by an autocatalytic mechanism upon exposure to the hydrochloric acid (HCl) found in gastric juice (pH of 1.5–2.0), which activates pepsin [14]. Its primary function in protein hydrolysis is to breakdown aromatic amino acids (such as phenylalanine and tyrosine) from the N–terminus of proteins [15]. As a consequence, the MW of active pepsin is 36 kDa [16]. Nalinanon et al [17] also documented the activation of pepsinogen to pepsin from albacore tuna (*Thunnus alalunga*) stomach via intermediate process, in which the segment with MW of approximately 36.8 kDa was generated before pro-segment was completely removed. The first 15 N-terminal amino acids of the pepsinogen activation segment were FHKLPLIKGKTAREE [17]. The optimal pH and temperature for pepsin were 2.0 and 50 °C, respectively. The activity remains stable within the pH range of 2–5. After heating at temperatures up to 50 °C for 30 min, more than 85% of its activity was retained [17]. Pepsin was strongly inhibited by pepstatin A and SDS (0.05–0.10%, w/v), while E-64, ethylenediaminetetraacetic acid (EDTA), and soybean trypsin inhibitor had negligible effects [18]. Aliphatic alcohols and cysteine have been recognized as effective inhibitors of pepsin. In contrast, $CaCl_2$, $MgCl_2$, NaCl, molybdate, and ATP cannot inhibit pepsin [17].

Pepsin can be obtained from a wide range of sources such as human, fish, bird, sheep, and cattle [16]. Fish pepsins are primarily located in the stomach, although they are also present in the skin of puffer fish or the ovaries of brook trout [19]. There are various stomach pepsins, and they are all characterized by unique enzymatic properties and protein structure

[20]. Fish pepsins have been purified and characterized in several types of fish as displayed in Table 1. The amounts and types of PGs differed among fish species because each species has unique PG isoforms in its stomach [16].

Enzymatic activity of pepsin is reportedly influenced by three variables: inhibitor, temperature, and pH. Moreover, the activity of visceral proteases depends on the composition of the fish diet [21]. Each fish pepsin exhibits specific optimum temperature and pH stability [16]. Pepsin from warm water fish has a higher optimum temperature and can tolerate up to 40–50 °C. In contrast, pepsin from cold water fish is more sensitive to higher temperatures [16]. Pepsin has been employed in various applications including digestibility therapy, production of fish silage, use as a rennet substitute, medical application, and collagen extraction [22].

Extraction/recovery and purification of pepsin from fish viscera

A four-step process is used to separate the pepsin, which involves preparation of the fish stomach, extraction of crude PG, purification of PG, and activation of PG to pepsin at an acidic pH [33]. Highly purified pepsin is achieved through the conventional method, which includes several steps such as fractionation with ammonium sulfate, gel filtration, or anion exchange chromatography. Nalinanon et al [17] used a series of chromatographies, including Sephacryl S-200HR, Sephadex G-50, and DEAE-cellulose for purification of pepsin from the stomach of albacore tuna (*T. alalunga*). The yield of 0.5% and purification fold of 658 were obtained.

Aqueous two phase system (ATPS), which is based on two immiscible aqueous polymer–salt solutions [34], is used for partial purification of pepsin. Zhao et al [35] used polyethylene glycol (PEG) with varying MWs (PEG 1000, 1500, 3000, and 4000) and ammonium sulfate salt at various concentrations (16, 18, 20, 22, and 24%) for purification of pepsin from Red Perch (*Sebastes marinus*). ATPS comprising PEG 1500 and salt at 18% showed the highest recovery yield of 86.2%. Although new approaches to pepsin partitioning have been proposed, the industry still needs large-scale pepsin recovery. Pepsin is acknowledged as a promising protease with numerous uses in both traditional and industrial sectors. Thus, effective and promising technology for recovery of pepsin is still required. Stabilization is another factor determining the efficient application of pepsin from fish stomach, and Patil et al [36] stabilized pepsin from skipjack stomach using some cryoprotectants by dissolving the pepsin in 10% maltodextrin and 5% trehalose solution before freeze-drying (Fig. 1). It was found that pepsin was stable up to 4 weeks at different relative humidities, in which residual activity of approximately 75% was retained. Maltodextrin and trehalose-stabilized tuna pepsin was also active in hydrolysis of different proteins, including soy protein isolate, whey protein isolates as well as fish myofibrillar proteins [36].

Trypsin

Trypsin (EC 3.4.21), a serine protease, performs an important role in protein digestion [12, 21]. It has been extracted and characterized from the intestine, spleen, pancreatin, or pyloric caeca of various fish species [37]. Generally, serine proteases are defined by the existence of aspartic, histidine, and serine residues at their active site. Trypsin specifically features a catalytic triad comprising serine (Ser), histidine (His), and aspartate (Asp) within its S1 binding pocket. In fish, trypsins occur as isoenzymes with nearly identical specificities [37]. They break peptide bonds at P1 containing lysine and arginine residues. Fish trypsins are composed of a single peptide chain and have endopeptidase activity. MW of trypsin is generally 24 kDa [38]. However, MW of fish trypsins varies, depending on the species [37]. Trypsin, which has a high preference for lysine and arginine residues via the interaction of the negatively charged aspartic acid (Asp) in its S1 binding pocket with the positively charged P1 side chain of the substrate [39], exhibits high activity in the alkaline pH range of 7.5 to 11 [37, 40–42]. Trypsin cleaves synthetic substrates such as tosyl-L-arginine methyl ester (TAME) and benzoyl-L-arginine-ρ-nitroanilide (BAPNA) and is inhibited by serine-protease inhibitors such as aprotinin, soybean trypsin inhibitor (SBTI), and phenylmethylsulfonyl fluoride (PMSF). Fish trypsin exhibits less stability at acidic pH and possesses a lower number of basic amino acid residues in its polypeptide chain, compared to mammalian counterpart [12]. Therefore, trypsin completely loses its activity at very acidic condition [43]. Trypsins from spleen of skipjack tuna, yellowfin tuna, and tongol tuna had the optimal temperature in the range of 55–65 °C [44–46]. Furthermore, Patil et al [40] also found that trypsin from Asian seabass pyloric caeca had the optimal pH and temperature at pH 8.5 and 60 °C, respectively. Nevertheless, trypsin loses its activity at temperature above 70 °C, mainly due to thermal denaturation [40].

Extraction/recovery and purification of trypsin from fish viscera

The initial steps for recovering fish trypsins are: (i) extraction, which involves preparation of crude materials (entire viscera or separated organs rich in trypsin, e.g. pyloric caeca, hepatopancreas, intestine, spleen, etc.), extracting crude enzyme through homogenization in the selected buffer and centrifugation to separate the crude visceral proteases and (ii) fractionation or precipitation to collect proteases. Purification can be

Table 1 Optimal conditions of various fish pepsins and their isoforms.

Identified species	Molecular weight (kDa)	Optimum pH	Optimum temperature (°C)	Ref.
European eel (*Anguilla anguilla*)	30/30/30	3.5/2.5/2.5	40/40/35	Wu et al [23]
Snakehead (*Channa argus*)	32/33/31	3.0/3.5/3.0	45/40/40	Chen et al [24]
Skipjack tuna (*Katsuwonus pelamis*)	33.7	2.0	45	Nalinanon et al [14]
Rice field eel (*Monopterus albus* Zuiew)	32/32/31	3.5/3.0/3.0	40/40/35	Weng et al [25]
Rainbow trout (*Oncorhynchus mykiss*)	35/38/37	3.0/2.5/2.5	40/30/30	Wald et al [26]
Nile tilapia (*Oreochromis niloticus*)	NA	2.0	55	Martínez-Cárdenas et al [27]
Brazilian flounder (*Paralichthys orbignyanus*)	NA	3.5	45	Candiotto et al [28]
Sheepshead, (*Archosargus probatocephalus*)	NA	2.0	45	Merino-Contreras et al [29]
Lizardfish (*Saurida micropectoralis*)	32/31/30	2.0/2.0/3.5	40/50/40	Kuepethkaew et al [30]
Yellowfin Tuna (*Thunnus albacares*)	36	6.0	60	Osuna-Ruíz et al [31]
Palometa (*Pygocentrus nattereri*, Kner 1858)	NA	1-2	45	Medina et al [32]

NA: not available.

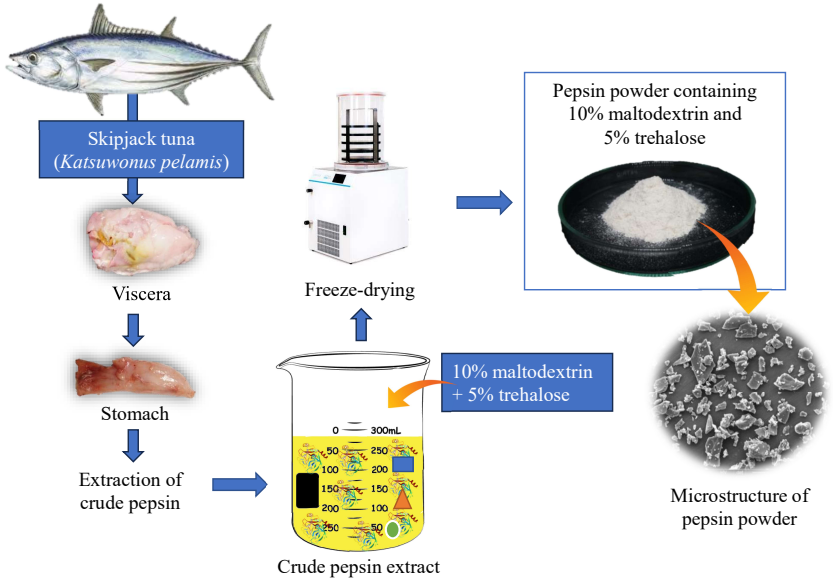

Fig. 1 Schematic representation of stabilization of pepsin from skipjack stomach using 10% maltodextrin and 5% trehalose before freeze-drying. Source adapted from Patil et al [36].

performed using ammonium sulfate precipitation at the appropriate concentration, followed by various column chromatography techniques, including affinity, ion exchange, and gel filtration chromatographies [47]. These methods are often combined to increase the enzyme purification; however, the selected methods depend on specific or target application [37]. Klomklao et al [45] used a series of chromatographies to purify trypsin from skipjack tuna spleen. Those included Sephacryl S-200, Sephadex G-50, and diethylaminoethyl-cellulose, respectively. However, it is tedious and time consuming. The use of affinity columns such as Sepharose 4B-trypsin inhibitor column has been widely used to purify trypsin due to faster processes. Trypsins from Asian seabass [40], bigeye snapper [48], and bluefish [41] have been purified using the affinity column after ammonium sulfate precipitation. MW, optimum pH, and temperature of trypsins from different fish are summarized in Table 2.

APPLICATIONS OF FISH VISCERAL PROTEASES

Pepsin

Pepsin is commonly employed for hydrolysis of proteins. It is utilized for the extraction of collagen [18, 64, 65] and gelatin [66]. It can be applied as a rennet alternative [66] and for digestibility therapy [36]. Fish stomachs, which make up 5% of the total weight of the fish, were used to recover pepsin [67]. Pepsin extraction and purification from fish and fish wastes have not been intensively focused. Therefore, the fish processing wastes, particularly stomach, can

Table 2 Optimal conditions of various fish trypsins.

Identified species	Molecular weight (kDa)	Optimum pH	Optimum temp. (°C)	Substrate	Ref.
Pacific cod (*Gadus macrocephalus*)	24	8.0	50	TAME	Fuchise et al [49]
Saffron cod (*Eleginus gracilis*)	24	8.0	50	TAME	Fuchise et al [49]
Hybrid tilapia (*O. niloticus* × *O. aureus*)	22	9.0	60	Casein	Wang et al [50]
Threadfin hakeling (*Laemonema longipes*)	24	8.0	50	TAME	Kishimura et al [51]
Brownstripe red snapper (*Lutjanus vitta*)	23	8.5	60	BAPNA	Khantaphant et al [48]
Barbel (*Barbuds callensis*)	24	10.0	55	BAPNA	Sila et al [52]
Hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*)	24	8.0	60	TAME	Klomklao et al [53]
Sardinelle (*Sardinella aurita*)	28.8	9.0	50–55	BAPNA	Khaled et al [54]
Silver mojarra (*Diapterus rhombeus*)	26.5	8.5	55	BAPNA	Silva et al [55]
Zebra blenny (*Salaria basilisca*)	27	9.5	60	BAPNA	Ktari et al [56]
Flatfish (*Paralichthys olivaceus*)	29.6	7.5	70	BAPNA	Kim and Jeong [57]
Mrigal carp (*Cirrhinus cirrhosus*)	21.7	7.6	30–40	BAPNA	Khangembam et al [58]
Catfish (*Luphiosilurus alexandri*)	24	9.0	50	BAPNA	Dos Santos et al [59]
Oil Sardine (*Sardinella longiceps*)	24	8.0	60	BAPNA	Khandagale et al [60]
Albacore tuna (*T. alalunga*)	30	9.0	55	Thrombin	Poonsin et al [61]
Common dolphinfish (*Coryphaena hippurus*)	26	8.0	50	BAPNA	dos Santos et al [62]
Asian Seabass (*Lates calcarifer*)	23.5	8.5	60	BAPNA	Patil et al [40]
Sardine (*Sardina pilchardus*)	27	8.0	60	Casein	Manni et al [63]

gain economic and environmental benefits from an efficient and cost-effective method of isolating and purifying pepsins from fish processing wastes. ATPS was used as a downstream technology to fractionate pepsin from crude extract [68].

Collagen extraction

Collagen is commonly extracted using an acid-solubilization method, where collagen is dissolved in an acid like acetic acid [69], and the non-acid-soluble components are subsequently filtered or centrifuged out [70]. Pepsin can effectively enhance collagen yield without compromising the molecular properties of the resulting collagen [71]. This is because pepsin has the capability to specifically cleave cross-linkages in the telopeptide regions of collagen without altering its secondary structure [18, 64, 70]. A general flow chart for the preparation of acid or pepsin soluble collagen is presented in Fig. 2. Fish pepsins have been demonstrated for significant improvement of collagen yield. Nalinanon et al [18] documented that the collagen yield from the skin of bigeye snapper (*Priacanthus tayenus*) increased to 18.7% when pepsin from the stomach of the same fish species was used, compared to a yield of 5.3% achieved with acid-soluble collagen (ASC). Ahmad and Benjakul [72] discovered that the collagen yields extracted from the skin of unicorn leather jackets (*Aluterus monocerous*) increased to 8.48% and 8.40% with the addition of pepsins from albacore and yellowfin tuna, respectively. This yield was greater than that of ASC (4.19%). Nalinanon et al [73] found that collagens with the yields of 74.48%, 63.81%, and 71.95% were extracted from the skin of threadfin bream (*Nemipterus* spp.) following a 12 h extraction using pepsins from albacore, skipjack, and tongol tuna, respectively. These values were higher than the yield of 22.45% obtained with ASC.

Medical uses

Pepsin is used to treat a variety of ailments such as infantile diarrhea, vomiting, gastralgia, dyspepsia, and some types of cancer, as well as to regulate digestion and act as a dental antiseptic [35]. Pepsin capsules and tablets have been produced in combination with HCl to boost the gastrointestinal tract digestibility and to increase patient appetites [74]. Pepsin is also added in animal feed to enhance protein digestion. Recently, Patil et al [36] proved that pepsin from stomach of skipjack tuna (*K. pelamis*) was able to replace commercial porcine pepsin and served beneficial supplement for patients with maldigestion, particularly the elderly. Tuna pepsin had comparable hydrolysis in simulated gastrointestinal tracts toward threadfin bream muscle protein, whey protein isolate, and kidney bean protein isolate to commercial pepsin, especially at a higher level (15 units/g protein). The peptides obtained from digestion by pepsin, which could pass through $CaCo_2$ monolayer, showed antioxidant activity [2]. Thus, fish pepsin can be used as digestion aid for the patient, who is suffering with maldigestion.

Cheese making

Animal (calf, cow, and pig) rennet is used in the production of commercial cheese and is made up of pepsin and chymosin, which are typically combined at a 1:9 ratio [16]. Nevertheless, the high costs of animal rennet have compelled the industry to seek new alternatives, and fish pepsin has emerged as a promising alternative [36]. Brewer et al [75] discovered that cod pepsin had a greater ability to coagulate milk, compared to calf chymosin at 15 °C, and sensory panel

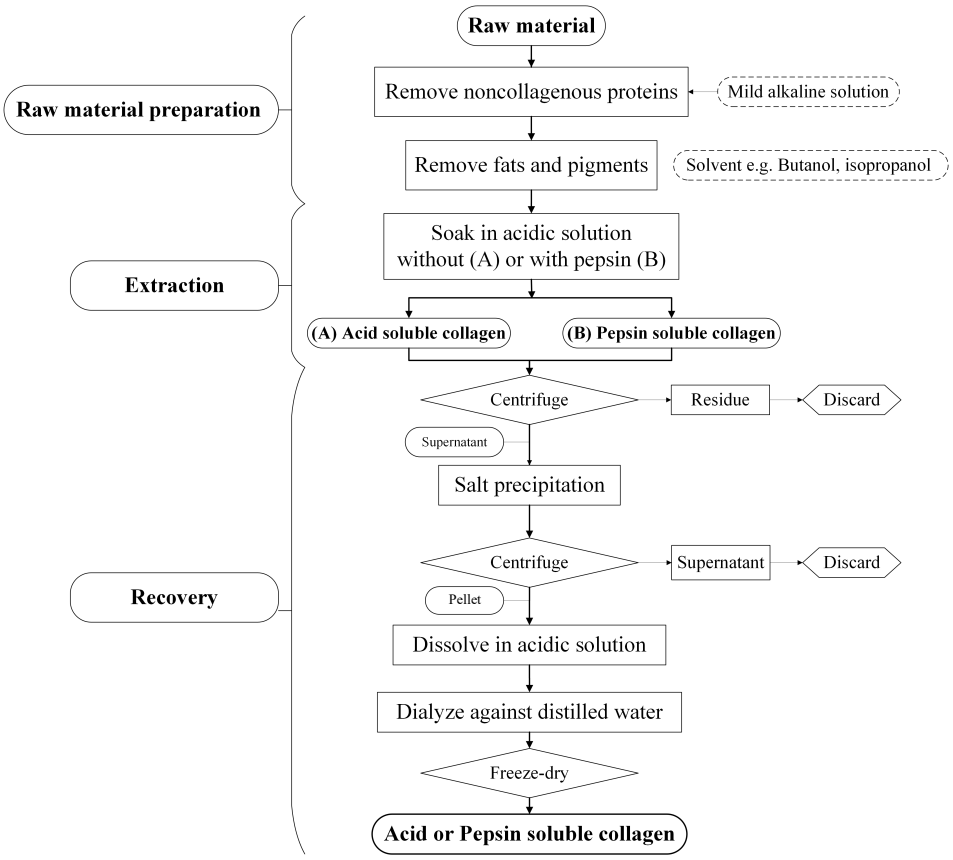

Fig. 2 General flow chart for the preparation of acid or pepsin soluble collagen.

revealed that the obtained cheese had a good flavor. Haard [76] reported that calf rennet has a higher temperature coefficient (2.2) for milk coagulation than cod pepsin (1.4). Tavares and Baptista [77] documented that tuna pepsin is effective as a commercial rennet in the pH range of 5.5–6.5 during cheese manufacturing. Despite these encouraging outcomes, fish pepsin-based cheese manufacturing has not yet been used commercially [78].

Fish silage

Fish silage is a liquid product created from minced fish and fish viscera that can be prepared with fish pepsin. Endogenous proteolytic enzymes function in the presence of acid to solubilize the fish components during this process [79]. Pepsin is one of the key enzymes in making fish silage [80]. Under acidic conditions, it can rapidly break down and liquefy minced raw material, mainly whole fish, into a very nutritious protein hydrolysate. Gildberg and Almas [81] reported that pepsins I and II in cod viscera were found to be useful in the development of aqueous cod viscera silage in acidic environments. Goddard and Al-Yahyai [79] indicated that fresh minced sardine (as a source of pepsin) produced an excellent silage when formic acid and propionic acid were combined.

Fish processing aid

Some fish products can be processed with the aid of fish pepsin. In New Zealand, caviar made from the roe of cold-water species like orange roughy (*Hoplostecthus atlanticus*) and Atlantic cod (*G. morhua*) has been produced using pepsin from these species [82]. Gildberg [80] reported that cod pepsin was utilized to descale haddock and hake under weak acidic condition. In addition, Joakimsson [83] used pepsin for herring deskinning. Furthermore, fish descaling using Atlantic cod crude pepsin has been marketed [80].

Applications of trypsin

There are several uses for trypsin in the food and beverage sectors. It has been used to make protein hydrolysates, tenderize meat, improve the texture of fish/mollusk meat, facilitate dough making, and increase beer cold stability [84].

Production of protein hydrolysate

Trypsin has been widely used for hydrolysis purposes. It is able to cleave peptide bonds of various proteins from animal and plant origins. Zamani and Benjakul [42] purified trypsin from unicorn leatherjacket (*A. monoceros*) pyloric caeca and used it to prepare fish protein hydrolysates with antioxidative activities. Khantaphant and Benjakul [85] extracted trypsin from pyloric caeca of 3 fish species including threadfin bream (*Nemipterus marginatus*), bigeye snapper (*P. tayenus*), and brownstripe red snapper (*L. vitta*) to produce gelatin hydrolysate from the skin of brownstripe red snapper. Hydrolysate with degrees of hydrolysis (DH) of 15% exhibited the highest ferric reducing antioxidant power and radical scavenging activities (ABTS and DPPH). Sripokar et al [86] used trypsin from the liver of albacore tuna (*T. alalunga*) to produce protein hydrolysates from the muscle of starry triggerfish (*Abalistes stellaris*) with improved antioxidant and functional properties. Patil et al [40] extracted trypsin from pyloric caeca of Asian seabass and used it to produce hydrolyzed collagen.

Medical and related applications

In biochemistry, trypsin is used in the development of cell and tissue culture protocols [87]. In medicine, the test of trypsin activity serves as a specific and reliable diagnostic tool to monitor pancreatic function and its alteration: cystic fibrosis, chronic pancreatitis, etc. [88]. Patil et al [43] proved that trypsin from pyloric caeca of skipjack tuna (*K. pelamis*) can hydrolyze food proteins effectively in the absence of digestive enzymes in simulated gastrointestinal tract. Trypsin-loaded beads (TLB) were successfully prepared via chitosan/alginate ionotropic gelation by Patil et al [2] (Fig. 3). Proteolytic activities of TLB toward red kidney bean protein (RKB), threadfin bream surimi (TBS), and egg white protein (EWP) in varying simulated *in vitro* gastrointestinal (GI) tract conditions were studied. TLB at a level of 50% (w/w of proteins) effectively hydrolyzed RKB, TBS, and EWP in a simulated *in vitro* GI tract. Thus, TLB from skipjack tuna viscera could potentially be used for enzyme supplementation to help digest food-proteins. Food-derived bioactive peptides generated after GI digestion could assist in improving human health due to their antioxidant activity.

Production of virgin coconut oil (VCO)

VCO is generally produced from coconut milk via inducing the collapse of emulsion under mild conditions. VCO is rich in medium chain fatty acids (MCFAs), especially lauric acid (C14), etc. [89]. MCFAs are burned up immediately after consumption and therefore the body uses it instantly to make energy, instead of storing it as body fat (Enig, 1996). Lauric acid is converted into a very valuable compound known as monolaurin, which has antiviral and antibacterial properties [90]. It is therefore assumed that consumption of coconut oil may help to protect the body from infections. Recently, proteases (especially trypsin) from shrimp hepatopancreas were used for extraction of VCO [91]. Various trypsins have been reported in many commercially valuable crustaceans such as Pacific white shrimp, freshwater prawn [92], and Harpiosquillid mantis shrimp [93], from which potential trypsin can be extracted, recovered, and utilized in various applications such as preparation of hydrolysate and VCO. VCO produced with the aid of hepatopancreas of Pacific white shrimp trypsin had a similar property to commercial VCO [94]. Patil and Benjakul [95] comparatively studied the extraction of VCO with the aid of partially purified trypsin from seabass pyloric caeca and commercial trypsin. Furthermore, Patil and Benjakul [96] used protease (especially trypsin) from seabass pyloric caeca in combination with repeated freeze-thaw cycles to increase the production efficiency of VCO.

Extraction of carotenoprotein

Basically, carotenoids, especially astaxanthin, are associated with proteins in crustaceans, known as carotenoproteins. Those complexes play a major role in the coloration of shrimp, lobster, and other crustaceans [97]. Since the shell or other discards from crustacean processing wastes contain a high amount of carotenoprotein, enzymatic hydrolysis has been employed for recovery. Carotenoprotein was extracted by trypsin from albacore tuna spleen [98]. Nasri et al [99] extracted alkaline proteinases (especially trypsin) from *Serranus scriba* viscera and applied them to extract carotenoproteins. Carotenoprotein from by-product of banana shrimp (*Penaeus merguiensis*) was extracted using trypsin from viscera of rainbow trout [100].

Meat tenderization

Texture is an important quality attribute determining the acceptability of meat such as beef. Apart from mammalian meat, squid rich in collagen and connective tissue also has a tough mantle and tentacle. Giant squid meat (GSM) is widely consumed globally for its unique taste, but its meat is tough and rubbery, thus giving an undesirable attribute to consumers. Tenderization is required to improve its tenderness. Recently, Patil et al [101] used Asian seabass trypsin to treat the giant squid mantle. To enhance tenderness, GSM was pretreated with pulsed electric field (PEF) at 250 V for 5 min (PEF-GSM) and then immersed in partially purified trypsin (PPT) at various concentrations (0, 5, 10, and 20 units/ml) with the aid of vacuum impregnation (VI) (2 cycles). Subsequently, PPT-soaked GSM

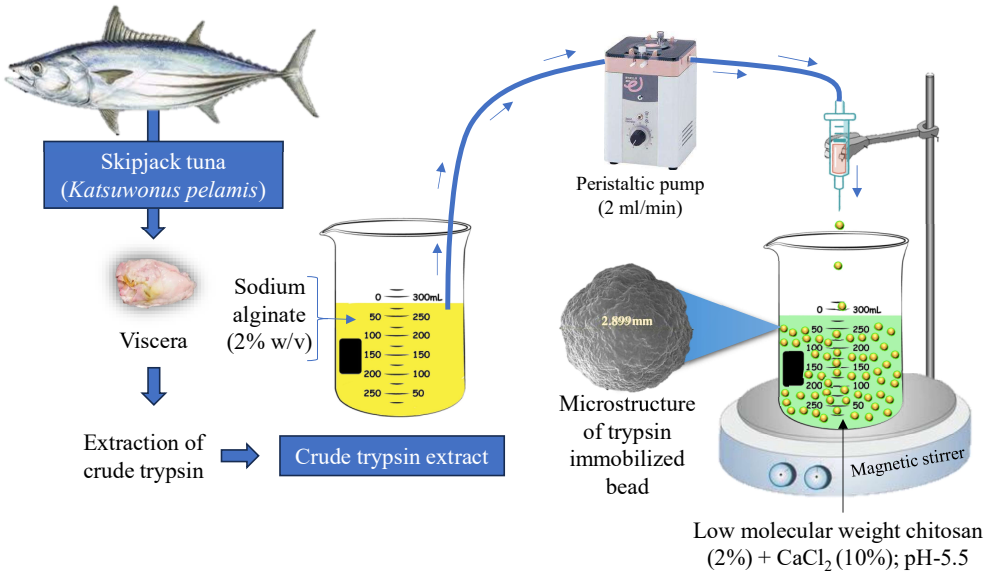

Fig. 3 Schematic representation of preparation of trypsin-loaded beads via chitosan/alginate ionotropic gelation. Source adapted from Patil et al [43].

was incubated at 60 °C for 5 and 10 min, followed by cooking at 100 °C for 2 min. The combined application of PEF, PPT (20 units/ml), and VI before incubation at 60 °C for 10 min significantly enhanced the eating quality of GSM, making it more preferable for consumers. Thus, fish trypsin can be further applied to tenderize the tough meat, e.g. beef and other meats, under the optimal treatment condition.

CONCLUSION

Fish viscera is an important source of various kinds of proteases existing in the fish and fish processing by-products, thus making it highly exploitable. These proteases possess a unique characteristic. Among the various kinds of proteases, pepsin and trypsin are the major proteases with a wide range of applications. Pepsin, a well-known aspartic protease, is present in the stomach, while trypsin can be found in several organs, especially pyloric caeca, hepatopancreas, intestine, spleen, etc. In the existing situation, the viscera can be exploited to recover the maximum amount of the aforementioned proteases for further applications in food, medical and pharmaceutical uses, etc. Thus, better management of waste from fish/shellfish viscera can make marine resources more sustainable and exploitable. In addition, several valuable products with marketable benefit can be produced.

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