Development of marine biotechnology as a resource for novel proteases and their role in modern biotechnology

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Abstract	1
Marine environment consists of the largest sources diversified genetic pool of material with an enormous potential for a wide variety of enzymes including proteases. A protease hydrolyzes the peptide bond and most of proteases possess many industrial applications. Marine proteases differ considerably from those found in internal or external organs of invertebrates and vertebrates. In common with all enzymes, external factors such as temperature, pH and type of media are important for the activity, catalytic efficiency, stability and proper functioning of proteases. In this review valuable characteristics of proteases in marine organisms and their applications are gathered from a wide literature survey. Considering their biochemical significance and their increasing importance in biotechnology, a thorough understanding of marine proteases functioning could be of prime importance.	2 3 4 5 6 7 8 9 10
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1. Introduction

The history of proteolytic enzymes or peptidases can be traced back at least to the late eighteenth century. However, in recent times the research work in this area has accelerated greatly, fuelled by numerous practical applications in biotechnology, and the realization of the fact that they are among major therapeutic targets [1]. The basic definition of a protease states that they are enzymes that hydrolyze peptide bonds in proteins. Classification of proteases is a hierarchical one built on the concepts of catalytic type, clan, family and peptidase. Proteases break the long chained molecules of proteins into shorter parts as demonstrated in Fig.1 [2]. Proteases are divided into two groups according to their mode of action: endopeptidases and exopeptidases (Fig. 2). Endopeptidases hydrolyze peptide bonds in the middle of polypeptide chains while exopeptidases remove terminal amino acid residues from polypeptide chains [3].

Alternatively, proteases may be classified by the optimal pH as neutral, acidic or alkaline as presented in Fig.3 [4]. With respect to their active center they could be classified into cysteine, serine, metallo, and aspartyl proteases (Fig. 4) [5]. About 75% of industrial enzymes are hydrolytic enzymes, representing one of the three largest groups of industrial enzymes allocated about 60% of the total worldwide sale of enzymes (Fig.5). A number of industries that use proteases include detergents, leather processing, silver recovery, medical purposes, food processing, feeds, chemical and waste treatment [6, 7]. For industrial application of proteases different temperature, salt concentration, optimal pH, type of media and incubation period are needed to be taken into account [4]. Proteases contribute for use in products that require the enzyme-aided or digestion of proteins from different sources [8]. Proteases that are consumed in the pharmaceutical industry differ from those used in food and detergent industries. In the pharmaceutical industry, proteases are produced in small amounts and extensive purification is required before they can be introduced to the market. On the other hand, they are prepared in bulk quantities and

used as crude preparations for other industries [5]. A number of sources are available to produce the enzymes including microorganisms, fungi, plants and various animals. However, much more efforts are directed towards extremophiles and symbiotic microorganisms. Fishes, prawns, crabs, snakes, plants and algae possess a wide range of biodiversity, although the most current bioprospecting activity is founded in microbial products [9]. It is a fact that optimal activities of enzymes in different organisms are related to salt concentration, pH and temperature as well as the interactive effects of these environmental factors. Moreover, a marine derived enzyme may carry novel chemical and stereochemical properties as compared to other rich sources. Biocatalytically oriented studies (suitable substrates, appropriator conditions, stereochemical assessment of catalysis) should be performed to reveal this "chemical biodiversity" which, in turn, increases interest for these enzymes [9]. In the last few decades, marine enzymes have been suggested for many industrial applications i.e. in pharmaceuticals, cosmetics, nutritional supplements, molecular probes, food additives, fine chemicals and agrichemicals [10, 11]. In particular, the use of sea-derived enzymes in food technology is becoming a promising application for the development of new processes and new products. Some examples are substitution of rennet in cheese manufacture; removal of the oxidized flavor from milk; ripening and fermentation of fish products; and preparation of fish protein hydrolysates and concentrates [12, 13]. The ability to maintain enzymatic activity at low temperature has made the digestive enzymes from marine organisms to be useful in food processing, As they can resist bacterial contamination and unwanted chemical reactions [14, 15].

In the present review an attempt is made to mainly compile an inclusive report covering classification, characterization and properties of proteases in different marine organisms such as, fishes, crustaceans, algae, acidians, bacteria and fungi.

2. Protease enzymes

2.1. Protease in marine bacteria and fungi

The sources of proteases in microorganisms are more than plant or animal kingdoms [16]. It has been reported that microorganism comprise of nearly 60 % of the total worldwide microbial protease market [17]. Their rapid growth, wide range of biochemical diversity, limited space required for cell cultivation and their various applications, are all contributed to the advantages of microbial sources as suitable pathways for large scale production of proteases [4]. Some divalent cations such as Mg^{2+} , Ca^{+2} are required for enhancing the activity of bacterial proteases, while other ions such as Hg^{2+} , Zn^{2+} , Fe^{2+} , Ag^{2+} perform inhibitory effect on the activity of microbial proteases [18-23]. However, a wide range of fungi such as genus *Aspergillus*, *Mucor* and *Rhizopus* and bacteria including genus *Clostridium*, *Bacillus* and *Pseudomonas* are potential sources to produce proteases [24, 25]. A mainly nutrient alkaline extracellular protease, present in genus *Bacillus*, has been recognized as sources of commercial protease and used in detergent, surfactants, builders, bleaching agent, bleach activators, fillers, fabric softeners and various other formulation aids due to increased production capacities, high catalytic activity and high degree of substrate specificity [8, 26]. Alkaline proteases possess many applications in food complementary of beasts and poultries, bakery, leathering, oil manufacturing, alcohol production, and beer production industries [4]. Some of bacterial alkaline proteases with major application as detergents are subtilisin Carlsberg, subtilisin BPN' and savinase which are also available commercially in market [27]. Another marine bacterium, *Pseudo alteromonas* strain A28, has been reported to produce an extracellular serine protease with the ability to destroy the diatom *Skeletonema costatum* strain NIES-324 cells due to its potent algicidal activity [28]. A facultative alkalophile *Bacillus clausii* could produce a kind of alkaline serine protease used as detergent additive to remove protein-containing spots from laundry [29]. *B. clausii* protease can exhibit suitable stability towards both surfactants and oxidizing agents, retaining its activity of 73 and 116% after incubation for 72 h with 5% SDS and 5% H_2O_2 . It can, therefore, be useful as additive in industrial applications including detergent preparations [30]. Hwang et al., have reported two *Escherichia coli* bacterial ATP-dependent proteases: while protease Ti is absolutely specific for ATP or dATP with two subunits of P and A, protease La can cleave and function with other nucleotides [31]. It

has been reported that the specific activity of protease Ti against casein is quite high; for component P, the activity appears to be about 10 fold higher compared to protease La [32]. The optimum pH and temperature of marine bacterium *Pseudo alteromonas* sp. strain A²⁸ have been reported to be 8.8 and 30°C [28]. The optimum pH and temperature for growth and protease productions of *Staphylococcus* are 8-10 and 37-45°C respectively [4]. However, the optimum temperature for proteolytic activity of protease producing bacteria is reported to be 37 -50°C [33]. The optimum conditions for *Bacillus cereus* enzymatic maximum activity are at 50°C and pH 10 [29]. Joo et al. have found a new strain of *B. clausii* that produce high levels of an extracellular alkaline protease with optimal pH 11 and temperature of around 60°C [30]. *Arthrobacter ramosus* and *Bacillus alcalophilus* is used for removing blood stains from cotton fabric due to their high protease activity. The enzyme is thermostable, ie., could remain stable at pH 12 and active in the presence of commercial detergent, suitable for use in detergent formulations [34]. The type of microorganism, together with chemical and physical parameters can affect proteolytic activity of enzymes. Thus, for good proteolytic activity identification and selection of potential microorganisms and optimization of physical and chemical conditions for protease producing isolates is definitely needed [4, 33]. In this regard the temperature, pH and the type of media used for potential protease producing microorganisms should be considered as a primary and important task. Maximum activity of a protease in optimum temperature and pH in some marine bacteria and fungi are presented in Fig.6. It has been indicated that parameters like temperature and pH affect the activity of enzyme and most species show their highest activity at 40°C. A group of researchers have tested the optimum pH for many samples and reported that for all samples to be 7- 7.5 depending on species, with maximum activity at pH 7.2. It has been shown that protease activity in *Bacillus clausii* [30] is higher than *Aspergillus niger* [25], *Bacillus clausii* [29], *Aureobasidium pullulans* [35], *Bacillus alveayuensis*

[36], and *Bacillus cereus* [4]. These results indicated that for the growth of protease producing bacteria and fungi, alkaline environment is more suitable.

2.2. Protease in thermophile microorganisms

The thermophile microorganisms are classified into several groups depending on their optimum temperature: facultative thermophiles (survive below 45 °C), thermo tolerant (grow at temperatures greater than 45°C), moderate thermophiles (grow at temperature between 45 and 60°C), strict thermophiles (grow at temperatures between 60 and 90°C) and extreme thermophiles or hyper thermophiles (grow best at temperatures greater than 90°C). Thermophilic microorganisms inhabit in different area, such as hot springs, geothermal sediments and marine solfatares or fermenting compost as well as in industrial environments, e.g. hot water pipelines [37]. The thermophilic marine microorganisms have several groups including phototrophic bacteria, bacteria domains and the archaea domains [38]. They belong to the genus *Bacillus stearothermophilus* (80°C), *Pyrococcus furiosus* (100°C), *Bacillus* SP.(60°C), *Thermus* SP.(55-85°C) and *Desulfurococcus kamchatkensis* (65-87°C). Almost all hyper thermophiles are archaea and they often grow at anaerobic conditions [39]. *Sulfolobus acidocaldarius* is an archaea that is active at 80°C which is a good example of a polyextremophile [40]. *Thermocrinis ruber*, an aerobic facultative chemolithotrophic bacterium, grows in alkaline hot spring at 44-89°C. An anaerobic autotrophic methanogens is *Methanococcus jannaschii* which grows optimally at 89°C in deep-sea hydrothermal systems [40]. Thermozyms are thermostable proteases from thermophilic and hyperthermophilic microorganisms that are stable and active at temperatures above 60-70°C [41]. Thermophilic proteases are suitable for bioengineering and biotechnological applications due to their higher resistance to chemical denaturants and organic solvents [42]. The ability to maintain stable folds in proteins at high temperatures is a specific property of thermophilic organisms importantly required for their adaptations [40]. A number of thermophilic and hyperthermophilic proteases are now used in

molecular biology and biochemistry research. For example, the hyperthermophilic serine protease extracted from *Thermus strain* Rt41A can be used in DNA and RNA purifications procedures. This enzyme can be used as an adjunct for PCR to break down cellular structures prior to PCR procedure and its optimal activity is at 90°C. A second hyperthermophilic protease S, extracted from *P. furiosus*, can be used in protein fragmentation for sequencing with optimal activity at 85–95°C [43].

2.3. Characterization of proteases from marine algae and yeast

A number of enzymes extracted from different marine algae show positive results for protease assay [44, 45]. Their activity varied with different species, temperature and pH (Fig 7 and 8). These properties could indicate that marine algae are a valuable source of proteases. However, very few studies are about alkaline protease of marine yeasts [44]. The optimal pH and temperature of the alkaline protease from the marine yeast *Aureobasidium pullulans* have been found to be 9.0 and 45°C, respectively. Studies have shown that the enzyme is activated by Cu^{2+} and Mn^{2+} and inhibited by Hg^{2+} , Fe^{2+} , Fe^{3+} , Zn^{2+} , and Co^{2+} [45]. The use of proteases from *Bacillus sp.* and lactic bacteria [46- 48] are most common. On the other hand, alkaline protease from the marine yeast *Aureobasidium pullulans* has potential uses in production of bioactive peptides from shrimp (*Trachypenaeus curvirostris*) muscle and spirulina (*Arthospira platensis*) powder [45]. Fibrinolytic and fibrinogen clotting enzymes are two specific proteases with fibrinolytic activity found in a number of genus *Codium* including *Codium intricatum* (CIPs) [49]. These properties are in common with examined fibrinolytic enzymes from other species of *Codium*. It has been shown that *C. diarticatum* possesses both fibrinolytic and fibrinogen clotting enzymes [50]. According to researchers, fibrinogenase is very useful for therapeutic application [50-51]. Damare et al. have studied the protease from deep-sea fungi, *Aspergillus ustus*. Their results have suggested that the enzyme of *Aspergillus ustus* is a serine protease because of its totally inactivation by PMSF (inhibitor of serine proteases). These enzymes belong to Group II enzymes because they are heat-

sensitive and relatively more active than mesophilic enzymes at a low temperature [52]. Optimum pH of *Aspergillus ustus* is reported as 9.0 showing about 25 and 45% of its maximum activity at 15 and 20 °C, respectively. Thus, fungi from deep-sea sediments could be a useful source of proteases. Protease in multi cellular than in unicellular algae plays an important role. In contrast to other organisms and higher plant proteases, the protease of microalgae has shown higher activities in the natural and alkaline ranges. The proteases from macro algae are highly variable, possibly due to responses to different environment conditions [53]. Reaction to stress conditions can induce synthesis of specific proteins and enzymes. Stressful conditions can also affect protein stability and increase the rate of proteolysis of some proteins [53]. It has been found that proteases from brown seaweed such as *Scythosiphon lomentaria* and *Ecklonia cava* show strong antioxidant activity. The enzyme is soluble in water and the large scale production process of antioxidant extracts from seaweeds is quite simple [54].

2.4. Proteases from polychaeta

The polychaeta are a paraphyletic class of annelid worms, generally marine. They are robust and widespread from within species that live in the coldest ocean temperatures of the abyssal plain, to species which tolerate extremely high temperatures near hydrothermal vents. *Capitella* sp. I and *Perinereis nuntia brevicirris*, are two polychaete living in different coastal habitats. *Capitella* sp. I, is found in sediment under a fish farm, while *P. nuntia brevicirris* which inhabits estuaries [55]. The proteases extracted from two polychatae have shown activities adapted to their habitat. In addition, these polychaetes produce the specific proteases by themselves [55]. In their habitants, the relationship between *Capitella* sp.I and bacteria is symbiosis, because this polychatae has markedly enhanced bacterial growth and bacteria could decompose the organic matter within the sediment [55]. *Capitella* sp. I may consume protein-rich organic material and, depending on to the food type, it needs high

protease activity whereas *P. nuntia brevicirris* requires high cellulase activity to become well adapted to living in an estuary [55].

The proteases of *Sabellaria alveolata* can be classified into five groups, i.e., T protease (the most abundant in digestive fluid), C4 protease (very abundant in digestive fluid and is a typical chymotrypsin-like enzyme), C2 and C3 proteases (very close to chymotrypsin but are not abundant), proteases hydrolyse trypsin and chymotrypsin substrates equally well and the last one is very similar, but with lower esterolytic activities [56].

Thromboembolism is a lethal medical intricacy and thrombolytic agents used for treatments of thromboembolism intricacy [57]. Many organisms such as insects, earthworm, leech, marine green algae and microorganisms are important source of thrombolytic agents. *Nereis* (Neanthes) *virens* (Sars) are common intertidal marine polychaete (Annelida: Polychaeta) with a novel fibrinolytic serine protease from their coelomic fluid possessing a strong fibrinolytic activity. Thrombolytic agents generate plasmin from plasminogen suggesting that the availability of this protease from clamworm may be used as an attractive alternative approach for thrombolysis treatments [58]. A novel protease, extracted from the polychaeta, *Periserrula leucophryna*, is a novel alkaline protease having a strong stability against extreme of pH and temperature, detergents, oxidizing agents, heavy metals and another solvents that make it suitable for industrial targets [59].

2.5. Proteases of crustaceans

Crustaceans are among the species that feed on animal tissues of collagen content. However, little attention has been made on these species. A collagenolytic protease enzyme has been obtained from the hepatopancreas of the fiddler crab, *Uca pugilator* [60]. As with collagenases in vertebrate collagenolytic protease, the enzyme is extractible in its active form [61] and with other collagenases this enzyme acts

on native collagen in solution and is characterized by the cleavage of the native collagen helix at loci 75, 70, and 67% from the amino terminal of the molecule [61]. In purification of collagenolytic protease, persistence of enzymatic activities and specificities are normally associated with trypsin and chymotrypsin [62]. The existence of such activities is not unexpected in view of the digestive function of the crab hepatopancreas. It has been reported that neither vertebrate trypsin nor chymotrypsin, in quantities equal to or greater than that present in hepatopancreas extracts, is capable of cleaving the native collagen helix in either soluble or fibrillar form [62]. The collagenolytic protease in crustaceans differ from mammalian trypsin in a number of its properties such as, are acidic in nature, inactivation at acid pH, lack of stabilizing by calcium, amino acid composition and strong affinity for DEAE-cellulose [63]. However, in these characteristics the enzyme is similar to a number of other isolated and reported invertebrate digestive proteases [63-65]. Despite similarities and differences, it is important to understand whether the hepatopancreas collagenase from *Uca pugilator* is a member of either the family of vertebrate serine proteases (shown by primary structure homology to be related to proteolytic enzymes in lower invertebrates) [66] or to the serine protease family represented by the subtilisins (similarity of mechanistically but unique of structurally) [67]. Thus, collagenolytic protease may represent yet a third class of enzymes that function via active serine residues but is unrelated by obvious evolutionary pathways to either of the other two families. For all of differences, it is obvious that the hepatopancreas collagenase is related to the vertebrate serine proteases [63]. Distinction of the amino acid composition of the collagenase from *Uca pugilator* hepatopancreas to other serine proteases is in clear similarity between the crustacean collagenase and starfish trypsin, an enzyme which has been suggested to bear an evolutionary relationship to bovine trypsin [63]. It has been found that, the presence of amino-terminal aspartic acid in this enzyme would imply that does not exist as a zymogen, which is in agreement with direct measurements [62]. Eisen and Jeffrey in 1969 reported that the collagenolytic

enzyme from the hepatopancreas of *Uca pugilator* is capable of degrading the polypeptide backbone of the collagen molecule under conditions that do not denature the protein [62]. The hepatopancreas collagenase in *Uca pugilator* is a digestive rather than a morphogenetic enzyme in other animal collagenases. It cleaves collagen molecule at the end of TC^{A75} [61] producing numerous smaller fragments in a shape similar to that of certain other tissue collagenases [61- 68]. Protease in invertebrates and vertebrates are shown in Table 1. It is indicated that the proteases in Freshwater crab, Cry fish, King crab and Fiddler crab are collagenases enzyme, while chymotrypsin and trypsin represent proteases in Scallop, White shrimp and Krill. The first reported of invertebrate trypsin enzyme were published in the 1960s belonging to insects and crustaceans [69]. It has been suggested that invertebrates synthesized paralogue and arthologue trypsins (the means of paralogue is homologue enzymes in the same organisms and orthologue is homologue enzymes within taxa) [70]. Trypsin is different in invertebrates depending on the feeding regimes and their habitants. These properties are impacted on their life styles, survival ability and their environments [71]. Kinetic parameters of trypsin from fish and crustaceans are presented in Table 2. The lowest catalytic efficiency, k_{cat}/K_m , of PaT4 is in lobster (*Panulirus argus*) 2.02s⁻¹mM⁻¹. It is important to note that trypsin and chymotrypsin from vertebrate sources are incapable of cleaving the native collagen helix. However, chymotrypsin is able to attack peptide bonds from the non helical region at the amino-terminal end of the collagen molecule at or near the site of the intra molecular cross-link [72]. The conversion of P to Q components by this collagenase is possibly related to its chymotrypsin-like activity. Future sequence studies designed to elucidate the structure of this enzyme will provide an opportunity to inquire further. This is one of the important serine proteinases is in several biological processes in higher animals such as digestion, proenzyme or prohormone, complement activation and blood coagulation [73-75]. Many serine proteinases consist of N-terminal propeptides and C-terminal serine proteinase domains [76]. Protease in crayfish blood cells

is a prophenoloxidase activating enzyme (ppA), kind of serine type proteinase. Its properties are sensitiveness to heat inactivation at 58°C and highest enzyme activity at natural and slightly alkaline pH [77]. Thus, the proteinase purified from crayfish haemo- cytes, which is able to mediate activation of haemo- cytic proPO, and designated as a prophenoloxidase activating enzyme (ppA), is the first ppA purified from the haemolymph of an arthropod [77].

It is worth indicating that other fundamental proteases found in crustacean (*Artemia salina*) are HEs. HEs help the encysted embryos to deliverance from their egg chorions during hatching process [78]. As compared to other specious, *Artemia* hatching enzyme shows pH optimal at 7.0 with optimal temperature of 40°C, summarized in Table 3. AHE is most probably a trypsin-type serine protease similar to HEs from shrimp *P. chinensis* [79], flounder *P. olivaceus* [80], killifish *Fundulusheteroclitus* [81], Zebrafish *Brachydaniorerio* [82], toad *X. laevis* [83] and mouse [84]. It however, differ from some fish HEs, a kind of trypsin-type alkaline protease [85, 86], frog *Ranapirica* HE, a kind of trypsin-type cysteine protease [87] and sea urchin *strongylocentrotuspurpuratus* HE, a kind of chymotrypsin-like protease [88]. A number of research studies have shown that it might be also a kind of Zn^{2+} -metallo protease as the proteolytic activity of AHE is almost completely inhibited by SBTI and p-APMSF, and greatly inhibited by TLCK, PMSF, and LBTI [Ref]. On the other hand, some studies have indicated that AHE might be also a kind of Zn^{2+} -metalloprotease, which is similar to that of HEs from marine sponge *H. pulcherrimus* [89], sea urchin *S. purpuratus* [88], sea-squirt *C. intestinalis* [90], shrimp *P. chinensis* [79], flounder *P. olivaceus* [80], medaka fish *O. latipes* [85], killifish *F. heteroclitus* [81], pike *Esox lucius* [91], toad *X. laevis* [83] and frog *R. pirica* [87].

2.6. Properties of proteases in fishes and aquatic invertebrates

In these species, proteases are used for protein catabolism in the muscle, formation of gonads in sexual maturation process and in deficit of eating condition as well as for building gonads (spawning

migration). It has been reported that culture conditions may change the enzyme content and change the proteolytic activity of enzyme [9]. The content of enzyme in muscles of marine animals differ from land animals and the free amino acid content in muscle from aquatic organisms normally range from about 0.5% (w/w) to 2% of muscle weight. Proteases found in vertebrates and invertebrates are shown in Table 2. The Atlantic mackerel (*Scomberscombrus*) and Atlantic horse mackerel (*Trachurustrachurus*), are important small pelagic fish species in many coastal areas of South Europe [92]. On the other hand, horse mackerel captured in West-European countries (Holland, Ireland, Spain, France, Germany, and Portugal) possess very important aspect of price and large quantities [93]. The reason for combination of physical, biochemical, and microbiological reactions is lack of fish freshness. Loss of enzymatic in fish is due to increased bacterial flora and demolition of muscle the postmortem of fish [94, 95]. The endogenous protease has a proteolysis role of fish myofibrillar proteins [96]. Cathepsins are one of the proteolytic systems of fish muscle during postmortem storage [97]. Following the breakdown, as the consequence of lysosomes, cathepsins B and D may be released from the lysosomal matrix into the cytoplasm and intracellular spaces [98]. The optimal activity for cathepsin B is at pH around 6.0 and between 3.0 and 6.0 for cathepsin D muscle tissue [99]. High pressure processing (HPP) is now commercially well-established in new food preservation technology replacing the traditional thermal treatments or chemical preservatives [99, 100]. Nowadays, HPP applications is very important in food processing because of the ability to inactivate food-borne microorganisms and endogenous enzymes [101] This is an important advantage as it results in preserving the nutritional and sensory attributes of processed foods [102]. However, HPP can affect on myofibrillar proteins [103], disrupt lysosomal membranes and release the fish muscle enzymes [99]. Studies have shown that HPP treatment causes an increase of cathepsin B activity at 500 MPa, while cathepsin D activity will increase after a pressure treatment below 300 MPa and decrease at higher pressures [104]. In cold-smoked salmon, however,

cathepsin B activity has been reported to be reduced by treatments at pressures of up to 300 MPa [105].
The applications of HPP suggest this technology useful for storage of fish as it minimizes various
alterations of fish products. However, the effect of HPP treatment on fish muscle enzymes with
proteolytic activity is still under investigation and poorly studied so far. Thus, the use of this technology
for improvement of frozen fish quality before storage at low temperatures has highly been suggested
[99]. It must be reminded that effects of HPP on cathepsins B and D depend on the pressure level and
time intensity [99]. As pepsins are not in invertebrates, cathepsin D acts both as a digestive and a
lysosomal enzyme in many species of these animals [106, 107]. The difference between fishes and
mammals cathepsin D are: 1) fishes with a true stomach have pepsinogen secretion to catalyze pepsin
2) fishes have higher pH optimum, very efficient at low temperatures and are less stable in strong acid
conditions 3) fishes are less thermo stable than mammalian pepsins [99]. In the case of haemoglobin
substrate [108 -110] the optimum pH of cathepsin D from fish and invertebrates are between of 2.8-4.0.
Optimum pH for a digestive proteinase from horse clam is 2.7 [111]. The optimum activity of cathepsin
D from the mantle muscle of a Pacific squid is reported at 35°C [112]. Whereas, an optimum temperature
as high as 60°C has been measured for highly purified preparations of cathepsin D from mussel mantle
and grey mullet muscle [106]. An optimum at 50°C was reported for cathepsin D from carp and tilapia
muscle [113, 114]. It has been reported that major activities of proteinase in mantle muscle of two squid
species are at pH optima at 2.6 and 3.6 and that cathepsin E was dominating in one species, whereas
cathepsin D was the major muscle cathepsin of the other species [115]. It has been reported that the pH
stability of cathepsin D from fish, squid and other invertebrates are very rare because the enzyme is
rapidly inactivated at a pH below 2 and very instable at a pH above 7.5 [107]. Thermal stability, pH and
K_m of proteases from fish and aquatic invertebrates are presented in Table 4.

2.7. Digestive proteinases from marine organisms and their applications

Proteinases found in the digestive organs of fish include pepsin, gastricsin, trypsin, chymotrypsin, collagenase, elastase, carboxypeptidase and carboxyl esterase [116, 117]. Marine animals are highly associated with properties of their proteinases. They are conformable with inter- and intraspecies genetic variations and environmental conditions [117]. Their properties also depend on the respective marine animals including higher catalytic efficiency at low temperature and lower thermal stability [118]. Various aspects of proteinase varies with different with organs and species they exist in them. Pepsin and trypsin are found in stomach and pyloric ceca and intestine respectively [119]. Besides, pepsin has an extracellular function as the major gastric proteinase. In rainbow trout, (*Salmogairdneri*) the activity of proteinase has been found to be higher than that of Atlantic salmon (*Salmosalar*) [120]. The proteinase activity in the intestine of discus fish (*Symphysodonaequifasciata*) is reported higher than that in stomach [121]. Fish digestive proteinases are unique compared to mammals and have different properties among fish species in various aspects of environmental conditions. This properties the to be very useful tools in the food industry as they have thermal instability and high activity at low processing temperatures. The Protease type S1A which is a serine protease is especially common in plants and protists, but rare in fungi. The distribution in bacteria is largely dependent on the genus Streptomyces and absent in Archaea [122]. In 2002, Rojas et al suggested that existence of a group of S1A serine in a sponge (Phylum Porifera) and Jellyfish (Phylum Cnidarian) make it safe. These enzymes, containing three or more disulfide bonds, are extremely abundant in both vertebrate and invertebrates [123]. The most important problem that seafood processors encounter is their transport and storage. Collagenous protein degradation and muscle softening in fish and shellfish by intensive hydrolysis of serine-like collagenolytic proteases during postmortem storage are to be mentioned. In order to so solve these problems, the use of natural serine or trypsin inhibitors is the most preferred way [124]. Cystatin is an inhibitor of protease with low molecular weight that can be diffused into intact fish muscle cells effectively to minimize proteolytic activity and meat softening [125].

Invertebrate trypsins belong to the Clan PA, family S1A and they play some distinct properties such as their dependence to calcium ions, activity towards native proteinaceous substrates, high number of isoforms, and low stability at acid pH (1). Trypsins are cold-adapted crustaceans with higher catalytic efficiency than those in the tropical lobster *P. argus*. Similarly, trypsins in fish such as salmon tend to be more efficient than their crustacean e.g. in krill counterparts [126]. Various aspects of trypsins isoforms in invertebrates and vertebrates reveal that invertebrates show a high number of isoforms for digestive trypsins, while lower vertebrates such as fishes still have various numbers of isoforms. It has been found that in *Daphnia magna* the expression and activity of trypsin isoforms in response to protease inhibitors in diet is quite different [127] which may be due to physiological role of multiple trypsins in crustaceans. The protease in fish muscle consists of cathepsins B (EC 3.4.22.1) (found in the sarcoplasm of muscles active against many myofibrillar proteins), calpains (EC 3.4.22.17), cathepsins L (EC 3.4.22.15) (found in the lysosomes of muscle fibers as well as in phagocyte cells) and collagenases (EC3.4.24.7) (found in skeletal muscle) which are summarized in Table 5.

3. Conclusion

Proteases have a long history of application in different industries which is expected to increase further in a very near future. In the biotechnological processes, proteases are ideal candidates for structure-function relationship studies. It is interesting to notice that marine animals are a main source of proteases with different conditions. They exist in their optimal conditions, stability and function among fish and aquatic invertebrates. Extracted protease from fish, as compared to mammals, is unique and these differences are chiefly related to the great range of environmental conditions. The proteinases in fish have thermal instability and high activity at low temperatures that highly contributes to their application in industry. One of importance and potential advantages of using enzymes from fish and aquatic invertebrates, as compared to mechanical or chemical methods, is the development of gentle enzymatic methods as

alternatives to mechanical or chemical treatments that cause damage to the product. Moreover, industrial scale recovery of marine enzymes is still under experimental stage. It is expected that expanding capabilities of this new area will continue to profoundly affect the fish and invertebrate industries in future. However, further research is still required to better understand the processing lines and to develop new techniques that may be tailored to the specific requirements of production of various products.

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Table1. Protease enzymes in marine vertebrates and invertebrates.

Specious	Protease enzyme	Reference
Shark	Pepsinogen	[128]
Rainbow trout	Pepsinogen	[129]
	Chymotrypsin	[130]
Atlantic cod	Gastrisin	[131]
	Chymotrypsin	[132]
	Pepsin(Gastric protease)	[133]
	Elastase	[134]
	Collagenases	[135]
Tuna(blue fin)	Pepsinogen	[136]
	Pepsin	[136]
Sardine	Pepsin(Gastric protease)	[137]
	Trypsin(Intestinal proteinases serine protease)	[138]
Caplin	Pepsin(Gastric protease)	[137]
	Chymotrypsin	[139]
	Trypsin	[140]
Catfish	Elastase	[141]
Spiny dogfish	Chymotrypsin	[142]
Herring	Chymotrypsin	[139]
Salmon	Pepsinogen	[128]
American smelt	Pepsin(Gastric protease)	[143]
Fiddler crab	Collagenases	[62]

Table 2. Kinetic parameters of trypsins from Fish and Crustaceans.

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Specious	Temperature(C)	kcat/Km(s ⁻¹ mM ⁻¹)	Reference
Spiny Lobster(<i>Panulirusargus</i>)	37		[126]
PaT1		4.82	
PaT2		5.02	
PaT3		4.98	
PaT4		<u>2.02</u>	
PaT5		6.04	
Slipper Lobster (<i>Thenusorientalis</i>)	37		[144]
TRY-TO		9.78	
Krill (<i>Euphausiapacifica</i>)	37		[145]
TRY- EP		9.5	
Chum Salmon(<i>Oncorhynchusketa</i>)	35		[146]
ST-1		110	
ST-2		135	
ST-3		96.5	
Anchovy (<i>Engraulis japonicas</i>)	25		[147]
aT-I		52	
aT-II		112	
Anchovy (<i>Engraulisencrasicholus</i>)	25		[148]
Trypsin A		1.86	
Trypsin B		4.84	

✓ The low catalytic efficiency of PaT4 (*Panulirusargus*) is underlined.

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Table 3. Properties of HEs in invertebrates and vertebrates.

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Specious	Molecular weight(kDa)	pH	Temperature Optimum(°C)	Km (mg/ml)	Reference
Sea urchins	37–51	8.0	35		[149]
Shrimp <i>P. chinensis</i>	43	8.5	40	7.47	[79]
Sea-squirt <i>Cionaintestinalis</i>	34	8.5	-	-	[90]
Fish	15–40	3.0–8.7	30–35	-	[85]
Shrimp <i>Artemiasalina</i>	73.3	7.0	40	8.20	[78]

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Table 4. Thermal stability, pH and K_m of fishes and aquatic **invertebrate proteases**.

Enzyme	Identified species	Optimum temperature(°C)	Optimum pH	K _m (mM)	Activation energy (Kcal/mol)	Reference
Protease I	Sardine	55	4.00	-	-	[138]
Protease II	Sardine	40	2.00	-	-	[138]
Pepsin I	Arctic capelin	38	3.70	-	-	[137]
Pepsin II	Arctic capelin	43	2.50	-	-	[137]
Pepsinogen	Rainbow trout	37	3.75	-	9.13	[129]
Pepsinogen A	Polar Cod	37	4.75	0.060	3.20	[150]
Pepsinogen B	Polar Cod	37	2.50	1.330	2.90	[150]
Protease I	Orange roughy	37	3.50	0.124	-	[151]
Protease II	Orange roughy	37	3.50	0.517	-	[151]
Pepsin	Palmoeta (<i>Paronassignata</i>)	37	3.00-3.50	-	-	[152]
Pepsin	Greenland cod	30	3.50	1.140	4.70	[153]
Pepsin	Greenland cod	-	3.00-3.50	0.860	-	[154]
Pepsin	Arctic cod	32	3.50	0.400	4.10	[153]
Pepsin I	Atlantic cod	40	3.00-3.50	0.175	-	[137]
Pepsin II a	Atlantic cod	40	3.00	0.033	-	[156]
Pepsin II a	Greenland cod	40	7.5	1.670	8.20	[157]
Pepsin II a	Greenland cod	40	7.5	0.260	7.00	[158]
Pepsin II a	Atlantic cod	40	7.5-8.0	1.480	12.80	[155,160]
Pepsin II a	Atlantic cod	55	8.0	0.077	7.57	[159]

Pepsin II a	Atlantic cod	40	7.5	1.480	8.90	[155 ,157]
Pepsin II a	Atlantic cod	43	8.5-9.0	-	-	[168]
Pepsin II a	Cunner	50	8.0	0.730	11.9	[155- 157]
Pepsin II a	Cunner	45	8.5	-	11.8	[161]
Pepsin II a	Atlantic salmon	45	8.5	-	-	[162]
Pepsin II a	Atlantic white Croaker	60	9.5	0.081	-	[175]
Pepsin II a	Palmoeta (<i>Paronassignata</i>)	65	8.5	-	-	[152]
Pepsin II a	Crayfish	50	6.0	-	-	[163]
Pepsin II a	Oyester	60	8.0	-	-	[164]
Pepsin II a	Hybrid tilapia	40	9.0	2.500	8.7- 8.9	[165]
Thymotrypsin	Atlantic cod	52	7.8	0.510	-	[159]
Thymotrypsin	Crayfish	50	6.0	0.226	-	[166]
Thymotrypsin	White shrimp	-	8.0	1.600	-	[167]

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Table5. Properties of proteases in fish muscle.

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Enzyme	Calpains (EC 3.4.22.17)	CathepsinsB(EC 3.4.22.1), CathepsinsL(EC 3.4.22.15)	Collagenases (EC3.4.24.7)
Properties			
1	A Neutral cysteine endopeptidases	A cysteine protease [172]	Is ametalloproteinase's
2	Most activeat neutral pH (6.9-7.5)[169]	Best activity is at pH(3-4) [174]	Are most active at pH values close neutrality (7-8) [171]
3	Most active at30 °C [169]	timum activity is at about 40°C - 50 °C[174]	Found in skeletamuscle [169]
4	Found in the sarcoplasm of muscles also are active against many myofibrillar proteins. [169]	Cathepsins are found in the sosomes of muscle fibers and in phagocyte cells	Play a role in the loss of integrity of the muscle structure in fish held at abuse temperatures [170]
5	Activated by calcium ions and thiol compounds [169]	High activity is at pH6. 0-6.5[174]	
6	Markedly higher in fish during spawning migration [173]	ortant for deterioration of muscle texture[172]	Are activated and stabilized by metal ions (Ca2+)and other activators (such as stress, injury, infection or heat) [171]

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Figure captions

Fig 1. The general mechanism of protease enzymes action. The Proteases cleave proteins by a hydrolysis reaction the addition of a molecule of water to a peptide bond.

Fig2. Classification of proteases, in terms the site they react. They are divided into exo- and endopeptidases according to their action at or away from the termini, respectively.

Fig3. Classification of proteolytic enzymes on the basis of their sensitivity to pH.

Fig4. Proteases are classified on the basis of catalytic mechanism, and five known distinct classes are described: metallo, aspartic, cysteine, serine, and threonine.

Fig5. Distribution of different enzyme sales.

Fig6. Maximum activity of proteases in optimum pH and temperature in marine bacteria and fungi. The maximum activity of *Bacillus clausii*protease was taken as 100.

Fig7. Effect of temperature (20–70 °C), on the protease activities in marine algae. The maximum activity of enzyme was taken as 100.

Fig8. Effect of pH on proteases activity in marine algae. 100% relative activity refers to the percentage of the initial reaction rate obtained by the enzyme at the pH value of maximum activity.

Figure 1

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Figure 2

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Figure 3

Figure 4

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Figure 5

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Figure 6

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Figure 7

Figure 8

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