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Extraction and characterization of proteolytic enzymes from fish visceral waste: Potential applications as destainer and dehairing agent

B. Sabtecha[1], J.Jayapriya[1]* and A.Tamilselvi[2]

[1]Department of Chemical Engineering, A. C. Tech,
Anna University, Chennai -600 025,India.
[2]Department of CHORD, Central Leather Research Institute (CSIR), Adyar,
Chennai-600 020, India.

*Corres.author: jayapriyachem@sify.com (J.Jayapriya),
Tel: +91 44 22359125; fax:+91 44 22350007

Abstract: Fish processing waste is an environmental contamination source. In order to explore the potential of fish processing wastes as natural resources for value added bioactive compounds, the visceral wastes of Red snapper,Seer fish, Great barracuda , Milk shark and Sardines were tested for their proteolytic enzyme activitiy. The results showed that higher proteolytic activity was observed for Red snapper and Seer fish (27.40±0.05 U; 23.21±0.61 U). The crude extract of the fish visceral waste was purified, using ammonium sulfate precipitation and sephadex G100 column chromatography. The optimum pH for protease in the fish viscera waste was 10. In addition, this enzyme showed its destaining capability against blood stained cloth and dehaired goat skins.
Keywords - Fish Visceral waste, Protease, Characterization, Destaining, Dehairing.

1. Introduction

Annually 100 million tons of fish catching happens around the world, out of which 60% is used for human consumption and the rest is utilized as fish meal[1]. The major by-products arising out of fish processing plants and fish markets include viscera, fins, scales, bones and muscle tissue and have long been considered as wastes. Majority of fishery byproducts are presently used for producing fish oil, fishmeal, fertilizer, pet food and fish silage[2,3]. However, most of these recycled products possess low economic value. Recent studies indicate a number of bioactive compounds (peptides, oligosaccharides, fatty acids, enzymes, water-soluble minerals and biopolymers) from fish bones and internal organs[4]. These bioactive compounds possess nutraceutical benefits for human and animal health. However, the high purity demands in these applications leads to high capital installation costs in downstream processing. In this context, downstream processing accounts for a larger cost share of the final product, and therefore, it is of increasing interest to study the direct applications of crude bioactive compounds from the waste generated in the fish processing plant. There are many physiological and biochemical differences between species, which translate into differences in the composition of fish waste components[5]. Therefore, in recent decades, marine scientists are looking into the composition of lipids, polysaccharides, enzymes, vitamins, and proteins in different fish visceral sources[6]. These approaches are in the research and development stages, and they provide an optimistic outlook for reclamation of fish waste into useful products.

Proteases constitute the most important group of industrial enzymes being used today, accounting for approximately 50% of the total industrial enzyme market[7]. They have diverse applications in a wide variety of industries such as detergents, food, pharmaceuticals and leather, and for the recovery of silver from used X-ray films[8-10]. Proteases are mainly derived from animal, plant, and microbial sources[11]. Fish viscera, is also known to be a rich source of digestive enzymes[12]. Hence, the objectives of this study are to isolate, purify and to characterize the protease from fish visceral waste generated in the fish market. It also aims to evaluate the performance of the crude and partially purified protease for different industrial applications.

2. Materials and methods

2.1 Fish sample and crude extract preparation

Visceral wastes of different fish species such as Red snapper (*Lutjanus campechanus*), Seer fish (*Scomberomorus guttatus*), Great barracuda (*Sphyraena barracuda*), Milk shark (*Rhizoprionodon acutus*) and Sardines (*Sardonella Longiceps*) were used. These visceral wastes were collected from the local fish markets (Chennai) in sterile polythene bags and stored at −20°C. These fish visceral wastes were washed thoroughly with distilled water and homogenized with 0.02M Tris- HCl, pH 8.0. The homogenate was centrifuged at 6000 rpm for 15 min and the supernatant referred to as "crude extract".

2.2 Protease assay and protein determination

The protease activity of crude extract was determined spectrophotometrically with casein as the substrate[13]. The crude extract (0.25 ml) was added to a tube containing 0.5 ml of 1% (w/v) casein (dissolved in 0.02M Tris-HCl buffer, pH 8.0) and incubated at 37 ^{o}C for 30 min. To this, 3 ml of a 5% (w/v) tri-chloroacetic acid (TCA) was added to stop the proteolysis. The mixture was incubated at 4 ^{o}C for 5 min. After incubation, the reaction mixture was filtered, and TCA soluble peptides in the filtrate were measured at 660 nm using Lowry's method. One unit of proteolytic activity (U) was defined as µg tyrosine liberated per ml per min of the enzyme extract. The protein content of the supernatant was estimated with the Coomassie blue dye binding, as described by Bradford[14].

2.3 Purification of protease

The crude enzyme was precipitated from the supernatant by the addition of ammonium sulphate, with gentle stirring until 80% saturation, followed by centrifugation at 10,000 rpm for 15 min. The pellet was dissolved in 0.02 M Tris-HCl buffer (pH 8.0) and dialyzed. Then the sample was passed through a Sephadex G-100 column equilibrated with 0.02 M of Tris-HCl buffer (pH 8.0)[15]. The fractions were collected at the flow rate of 0.5ml/min and analyzed for protease activity.

2.4 Determination of molecular weight

SDS polyacrylamide gel electrophoresis was performed on a 5% (w/v) stacking and a 12% (w/v) separating gel according to Laemmli[16]. The gel was stained with 0.1% (w/v) Coomasie blue for 2 h and then destained in 10% (v/v) acetic acid and 25% (v/v) methanol. A protein molecular weight marker (14-116 kDa) was used.

2.5 Effect of pH

To study the effect of pH, the protease activity was measured at different pH ranging from 4 to 11, using casein (1%) as the substrate. The pH of the reaction mixture was varied using different buffers: Citrate buffer (pH 4–5), Tris–HCl buffer (pH 6–8), and Glycine–NaOH buffer (pH 9–11).

2.6 Effect of temperature

The effect of temperature on protease activity was tested from 20 ºC to 80 ºC , using casein as a substrate for 30 min in 100mM glycine–NaOH buffer, pH 10.0.

2.7 Applications

2.7.1 Removal of blood stain

A clean piece of white cotton cloth (6 cm x 6 cm) was soaked in animal blood for 15 min and then allowed to dry. The stained cloth piece was incubated with enzyme extract for 20 minutes. A blood-stained fabric, washed with only tap water was taken as the negative control[17].

2.7.2 Dehairing

Common salt preserved goat skins were cut in to different small pieces (2.5cm x 2.5 cm) and incubated with the enzyme extract at 30±2°C. The skin was visually examined for complete dehairing activity, and changes in the skin morphology were evaluated using a scanning electron microscope (SEM) (Jeol,JSM-840 A).

3. Results and Discussion

3.1 Purification and characterization of Protease

Table 1 Protease activity of fish visceral wastes

Fish	Sample	Protein content (mg)	Protease activity (U)	Specific Activity (U/mg)
Red snapper	Crude	7.22± 0.21	27.40± 0.05	3.79± 0.19
	Ammonium sulphate precipitation	4.44± 0.37	42.20± 0.11	9.50± 0.12
	Sephadex G-100 purified sample	0.98± 0.21	57.63± 0.54	58.80± 0.31
Seer fish	Crude	9.44± 0.13	23.21± 0.61	2.46± 0.31
	Ammonium sulphate precipitation	7.22± 0.26	33.76± 0.37	4.68± 0.12
	Sephadex G-100 purified sample	4.44± 0.13	49.77±0.52	11.21± 0.19
Great barracuda	Crude	7.22± 0.05	10.9±0.99	1.51± 0.09
	Ammonium sulphate precipitation	5.00± 1.25	17.4±0.45	3.48± 0.35
	sephadex G-100 purified sample	0.98± 0.02	34.47±0.01	35.17± 0.18
Milk shark	Crude	7.22± 0.63	11.07± 1.88	1.53± 0.56
	Ammonium sulphate precipitation	6.89± 0.74	19.32± 0.96	2.8± 0.30
	sephadex G-100 purified sample	1.11± 0.21	29.34± 0.49	26.43± 1
Sardines	Crude	5± 0.77	11.6± 0.93	2.32± 0.43
	Ammonium sulphate precipitation	3.77±.001	24.38± 0.17	6.47± 0.03
	sephadex G-100 purified sample	0.98± 0.32	35.32± 0.27	36.04± 0.16

Protease production from visceral waste is strongly influenced by the genetic capability of the individual fish species (Table **1**). The visceral waste of Red snapper and Seer fish showed high proteolytic activity (27.40±0.05 U; 23.21±0.61 U) when compared with that of Great barracuda, Milk shark and Sardines. Protease from fish visceral waste was purified by the procedure described above (Refer Clause **2.1**). With 0–80% ammonium sulfate precipitation, the purity of protease increased almost two-fold (Table **1**). Then the precipitates were dialysed against 0.02 M Tris-HCl buffer (pH 8.0), for 24 h at 4˚C, prior to loading onto a Sephadex G-100 column chromatography. After purifying an enzyme by chromatography, there was a substantial increase in purification observed for the Red snapper, Milk shark, Sardines and Seer fish respectively (15.51±0.17; 23.29±0.38; 17.27±0.09; 15.54±0.19), the only exception being the Great barracuda (4.56±0.34).

The purity of the enzyme was confirmed by the SDS-PAGE (Fig. **1**). By SDS-PAGE, a single band with a molecular mass of 35 kDa, 34 kDa and 62 kDa respectively, indicates that protease extracted from the visceral waste of the Red snapper, Great barracuda and Sardines is a single polypeptide enzyme. Furthermore, two clear bands of proteolytic activity for the purified enzyme of Seer fish and Milk shark (32 kDa-50 kDa ; 20 kDa-35 kDa) were observed in Fig .**1**. It is noteworthy that alkaline proteases with molecular mass between 18 and 90 kDa have also been reported[18].

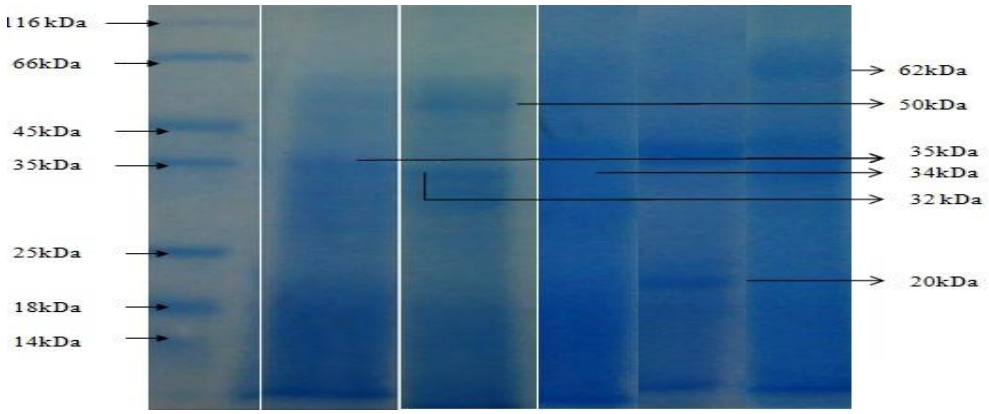

Fig.1. SDS- PAGE of purified protease. Lane 1- Protein marker; Lane 2 – Protease of Red snapper ; Lane 3- Protease of Seer fish; Lane 4- Protease of Great barracuda; Lane 5- Protease of Sardines ; Lane 6- Protease of Milk shark

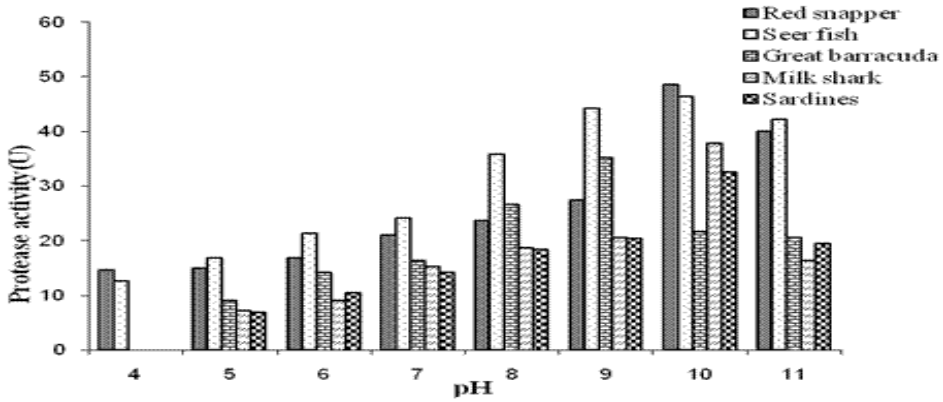

Fig .2. Effect of pH on protease activity

The effect of pH on the activity of protease from the fish visceral waste was determined over a pH range of 4.0–11.0 as shown in Fig.**2**. The optimal pH for the protease of the Great barracuda was found to be 9.0. However, the optimal pH for protease from other species was shown as pH 10.0. These results suggested that the protease from fish visceral waste was stable at alkaline pH and became unstable at acidic pH (Fig.**2**). Similar results were reported for Bolti fish (Tilapia nilotica) by El-Beltagy et al.,[15].

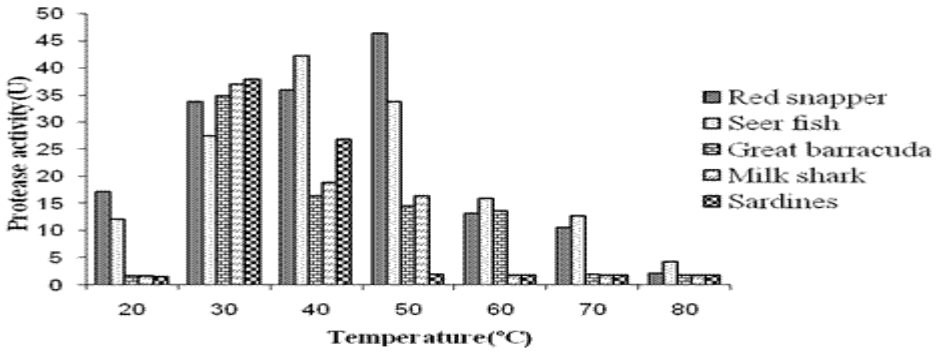

Fig .3. Effect of temperature on protease activity

Fig.**3** shows the effect of temperature on alkaline proteolytic enzymes from fish viscera. The protease was stable at temperatures ranging from 20 to 50˚ C, but enzyme activity was rapidly lost at temperature above 50˚C. On the other hand, it was noticed that the enzyme from the visceral waste of the Great barracuda, Milk shark and Sardines was inactivated at 40˚ C. The differences in thermal stability of an enzyme are governed by the fish habitat, environment and genetic features.

3.2 Removal of blood stain

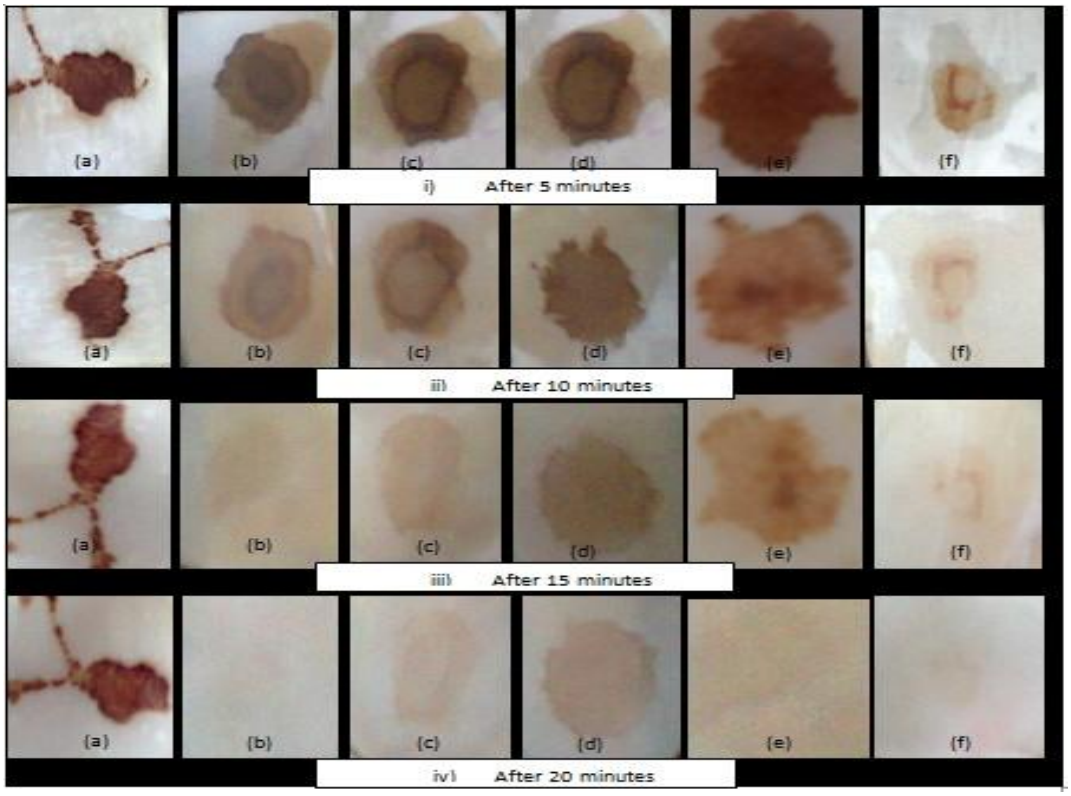

Fig.4. Effect of crude enzyme on the destaining of blood (a) Control (b) Red snapper (c) Seer fish (d) Sardines (e) Great barracuda (f) Milk shark

Table 2 Reflectance of blood stained cloth treated with crude protease extract from fish viscera

Fish type	% Reflectance of stained cloth
Control	4.93
Red snapper	67.39
Seer fish	65.78
Sardines	65.76
Great barracuda	68.72
Milk shark	63.92

Blood stained cloth was incubated at room temperature in crude and partially purified protease extract (15 U/ml) for different time intervals. As can be seen in Fig. **4**, the crude enzyme extract of the Red snapper and Great barracuda removed the blood stains effectively within 20 min, without the usage of any detergents. However, the partial removal of blood stains was seen when the cloth was treated with the crude enzyme of the Seer fish, Sardines and Milk shark. In agreement with this observations, the reflectance of the stained cloth treated with crude enzyme extract from the visceral waste of the Great barracuda and Red snapper was relatively high (Table **2**). The stain removal rate was found to be high as compared to control (Fig.**4**), which indicates that it could be used as an alkaline protease in detergent powder or solution. Interestingly, it was noticed that the alkaline crude extract from fish visceral wastes was more effective in destaining, when compared to partially purified protease (Fig.**4**).The direct applicability of the crude extract without downstream processing, would make its use acceptable as a substitute for commercial ones.

3.3 Dehairing of skin

The crude enzyme extract (15 U/ml) of the Red snapper, Seer fish, Great barracuda, Sardines and Milk shark dehaired the goat hide (2.5 x 2.5 cm^2) after 22 hrs of incubation, without the addition of sodium sulfide (Fig.**5**). There was almost no variation in the incubation period when the skin was treated with partially purified protease (data not shown). These preliminary results suggest that, there may be a good potential to utilize the

fish visceral waste for value added products. Several microbial proteases were evaluated for their dehairing property[19,20].The Epidermis was also removed after the incubation period of 16 h[21]. Comparing the dehaired skin with the untreated skin, it was found that the skin epidermis and hair bulbs were digested by the alkaline crude protease from the fish visceral waste in 22 h (Fig.**6**). This may be due to the degradation of proteoglycans. There have been relatively few attempts to recover proteolytic and collagenolytic activities from fish visceral waste[22]. Fishes are poikilothermic and vary considerably in their feeding habits and temperature preferences, and so it is expected that their digestive enzymes will also exhibit diversity[23]. The crude alkaline protease from fish visceral waste was considerably stable in a wide range of pH (Fig.**2**) and temperature (Fig.**3**), so that it has the potential for being a detergent additive and for dehairing of skin in leather industry.

Fig 5. Effect of protease from the visceral waste of Milk shark on dehairing A) Goat skin before dehairing B) Goat skin after dehairing

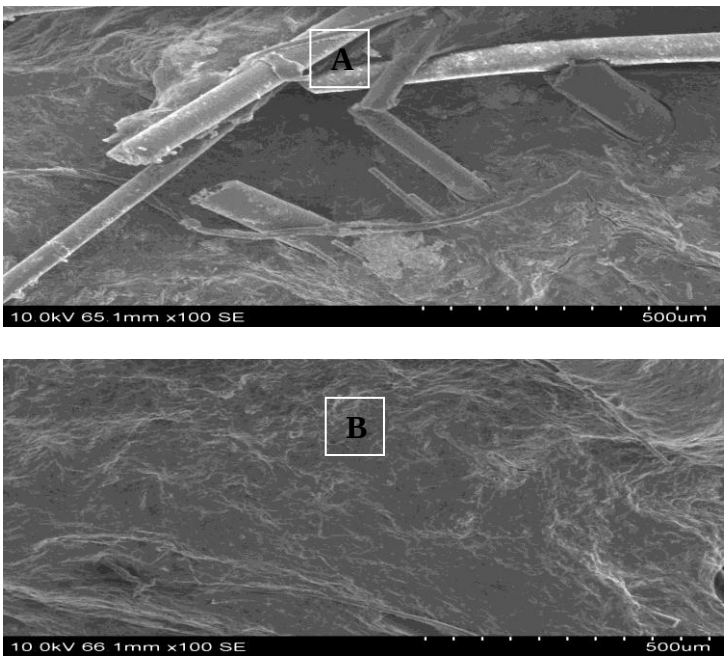

Fig. 6. Morphology of the goat skin A) Untreated B) treated with the protease of the Milk shark

4. Conclusion

The bioactive compound, protease, was extracted from the visceral waste of different fish species, and their characterization was studied. Each type of fish protease has a distinct optimum alkaline pH, temperature and molecular weight. The crude enzyme from the visceral waste of the Red snapper and Great barracuda removed the blood stains effectively within 20 min, without the usage of any detergents. It was also observed that the enzyme dehaired the goat hide after 22 h of incubation, without the addition of sodium sulfide. The direct applicability of the crude extract without downstream processing would make its use acceptable as a substitute for the commercial ones.

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