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Purification and Characterization of a Cysteine Proteinase from the Mid-Gut Gland of Indian Lobster, *Panulirus homarus*

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Abstract: A cysteine proteinase was isolated from the hepatopancreas of the lobster, *Panulirus homarus.* The enzyme was purified by a combination of ammonium sulphate precipitation, ion exchange chromatography and gel filtration. This enzyme was highly active at pH 8.0 and the optimum incubation temperature was found to be 60°C. It is a cysteine protease, both β-mercaptoethanol and dithiothritol stimulated protease activity. Investigation was conducted to evaluate the molecular weight of apparent proteases. Four protease activity bands were noted on the SDS-PAGE in between 27 and 45.5 kDa. The I and II protease band (27 kDa, 29.5 kDa) was belonging to cysteine group and these protein band was clear when the gel was zymographed with β-mercaptoethanol and dithiothritol.

Key words: Crustacean · *Panulirus homarus* · Lobster hepatopancreas · Cysteine protease · Zymography

INTRODUCTION

Enzymes are used as processing aids in the manufacture of food products to improve their quality, solubility and stability for centuries [1]. About 50% of the enzymes used as industrial processing aids are protein hydrolases which have been used in a number of industrial applications including laundry detergents, feed, leather treatment, silk degumming, cheese making, chill proofing, meat tenderizing, fermented sauces and the production of pharmaceuticals [2]. While, almost all living organism can be considered a potential source of useful enzymes, only a limited number of plant and animal tissues are economic sources and the greatest diversity of these comes from microorganisms [3]. In crustaceans, proteases enzymes are secreted into the digestive tract by specialized glands and are found in highly active concentrations [4]. In decapod crustaceans, the digestive gland is concerned with the digestion, absorption of nutrients, the storage of reserves and excretion [5]. The hepatopancreas or midgut gland is an organ that combines functions of the mammalian liver and pancreas and that produces digestive proteases [6]. It is also involved in the synthesis of digestive enzymes [7].

Most studies on digestive enzymes from decapods have focused on the classification of the catalytic mechanism [8]. Proteases such as cysteine proteinases have been isolated from a wide range of sources, including higher-plant fruit and latex, animal tissues and bacteria [9]. The food industry has capitalized on the properties of proteinases [10] and is currently beginning to seek large-scale, renewable sources of these catalysts [11].

Recently, interest has developed concerning the proteases found in stomachless marine organisms [12]. Also, the proteases have been characterized from various Lobster species namely, *Panulirus japonicus* [13], *Homarus americanus* [14], *Homarus gammarus* [6], *Panulirus interruptus* [15] and *Panulirus argus* [16]. In crustaceans the knowledge about cysteine proteinases is still limited [17]. Recently, digestive cathepsin D1 was isolated and characterized from the American lobster [18] and proteases from the hepatopancreas of fresh water prawn (*Macrobrachium rosenbergii*) [19] has been studied. The main objective of the present study was to characterize cysteine protease from the Lobster, *Panulirus homarus* for various biotechnological applications.

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MATERIALS AND METHODS

Animal: The lobster, *Panulirus homarus* was collected from the fish landing centres of Kanyakumari, South-Western coast of India. Live lobsters were taken to the laboratory for enzyme assay.

Sample Preparation: The mid-gut gland, hepatopancreas was dissected out and ground separately with Tris buffer (50 mM, pH 8.0) using a pestle and mortar. The sample was centrifuged for 10 min at 10000 *g* at 4°C. The resultant supernatant was separated and stored in 0.5 ml vials at-20°C. Suitable dilutions were made before performing enzyme assay.

Determination of Protease Activity: The reaction mixture consisting of 5 ml of casein (prepared in 50 mM of Tris buffer, pH 8.0) and an aliquot of an enzyme solution. The mixture was incubated for 30 min at 37ºC. The reaction was stopped by adding 5 ml Trichloroacetic acid solution (TCA) (110 mM). After 30 min, the mixture was filtered. To the two ml of the filtrate 5.0 ml of 500 mM of sodium carbonate and 1.0 ml of Folin-Ciocalteu's phenol reagent was added and kept for 30 min at 37°C. The absorbance of the sample was measured at 630 nm in a UV-Visible spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1µg of tyrosine/min under assay conditions [20].

Total Protein Estimation: Total protein estimation was done by Lowry *et al.* [21] method using Bovine Serum Albumin as standard.

Effect of pH on Enzyme Activity and Stability: The effect of pH on the protease activity was determined by incubating the reaction mixture at pH values ranging from 5.0 to 10.0, in the following buffer system (100 mM): succinate buffer (pH 5.0-6.0); sodium phosphate buffer (pH 7.0); Tris-HCl buffer (pH 8.0) and Glycine-NaOH buffer (pH 9.0-10.0). To evaluate the effect of pH on enzyme stability, the enzyme solution was mixed with buffer solution at varying pH and incubated at 37°C for 1 h. Later the protease activity was measured under standard assay condition. All experiments were conducted in triplicate and average values were reported.

Effect of Temperature on the Activity and Stability of Protease: The effect of temperature on crude enzyme was studied by assaying enzyme activity at different temperatures (30, 40, 50, 60 and 70°C) at pH 8.0 using casein as substrate. Thermal stability was measured through incubation of the protease in 50 mM Tris HCl buffer, pH 8.0, at various temperatures (30 to 70°C) for 30 min. The remaining activities after heat treatment were determined using 1% casein solution as a substrate at pH 8.0.

Effect of Divalent Ions on Enzyme Activity: To determine the effect of divalent ions on enzyme activity, the sample was incubated with divalent ions (5 mM) namely, Ca^{2+}, Mg^{2+}, Hg^{2+}, Zn^{2+} and Cu^{2+} for 30 min at 37°C. The relative enzyme activity was assayed under standard assay conditions.

Effect of Activators/inhibitors on Enzyme Activity: The enzyme sample was incubated with 5 and 10 mM of phenyl methyl sulphonyl flouride (PMSF), dithiothreitol (DTT) and β-mercaptoethanol along with the substrate (casein) and the percentage relative activity was assayed.

Protease Purification: Ammonium sulphate was added to precipitate the crude enzyme sample upto 70% of its saturation. After centrifugation at 10000 *g* at 4°C for 10 min, the pellets were resuspended in minimum volume of double distilled water and dialyzed overnight against the Tris-HCl buffer (pH 8.0) (3 times). The dialyzed sample was loaded on a pre equilibrated DEAE cellulose column. The column was washed with double bed volume of the same buffer to remove the unbound protein. The bound protein was eluted with buffer containing NaCl (0 to 1.0 M). The proteolytic fractions were combined, subjected to a sephadex G-75 gel filtration chromatography. This column was pre-equillibrated with 25 mM Tris-HCl buffer (pH 8.0) and eluted with the same buffer. The active fractions were subjected to electrophoresis.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis and Zymography: The proteins were separated by SDS-PAGE (10%) [22]. The sample was neither boiled nor treated with β-mercaptoethanol to maintain enzyme activity. After electrophoresis, gel was stained with 0.02% coomassie brilliant blue R-250 in an aqueous solution of 40% methanol and 7% acetic acid for 1 h and destaining with the same solution without dye. The half of the gel was used for zymogram analysis according to Garcia-Carreno *et al.* [23] with little modifications. Briefly, the gel was soaked in casein solution (3%, w/v) prepared in 50 mM Tris-HCl buffer (pH 8.0) for 30 min at 4°C and further incubated at 37°C for 1 h. To the casein solution, 10 mM β-mercaptoethanol was added inorder to activate the cysteine protease from the PAGE separated gel. The gel was further stained with coomassie brilliant blue R-250. Opaque zones on a blue background indicated endopeptidase activity.

RESULTS AND DISCUSSION

Effect of pH on Enzyme Activity and Stability: The optimum pH for protease activity was 8.0 (Fig. 1). Alkaline proteases of marine decapods crustaceans with similar pH range was reported [24]. In *Panulirus japonicus,* digestive proteinases was highly active at pH 7.5 [13]. These findings are in accordance with several earlier reports showing pH optima of *Penaeus orientalis* and the optimum pH range was 6.0 to 9.0 [25]. Similar result was reported in Norwegian lobster, *Nephrops norvegicus* [26]. In the present study, the protease was stable in the pH range of 7.0 to 10.0, however the enzyme was highly stable at pH 8.0 (100% relative activity). Enzyme activity was 63%, 75.5%, 81% for the pH 5.0, 6.0 and 7.0 respectively. The enzyme activity was decreased to 67%, 47.8% at pH 9.0 and 10.0 which was similar with the results of *Penaeus orientalis* [25].

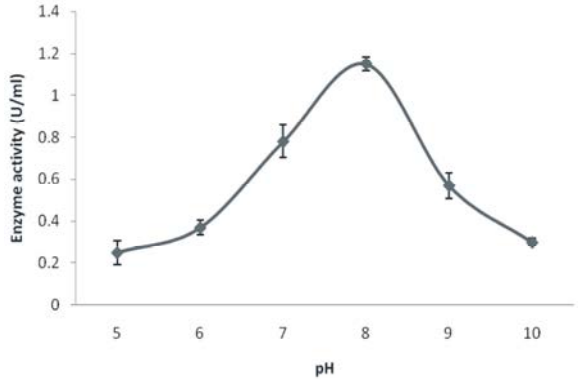

Fig. 1: Effect of pH on protease activity. The result was the mean of three different repeats.

Effect of Temperature on Enzyme Activity and Stability: The optimum temperature for protease activity was found to be 60°C (Fig. 2). Similar temperature optima had reported with the Japanese spiny Lobster, *Panulirus japonicas* [13]. The digestive protease of lobster has been found to have higher optimum temperature (60°C) [27]. The enzyme was stable up to 50 degree denaturing temperature (100% relative activity) and decreased thereafter. The enzyme stability was 91% and 97% at 30 and 40°C, respectively. The activity increased at 50°C may be due to the activation energy at this temperature. Enzyme stability was decreased dramatically to 41 and 37% at 60 and 70°C, repectively. These results were in accordance with Atlantic blue crab protease, whereas protease was stable at temperature ranging from 30 to 50°C for 30 min but activity was rapidly lost at 50°C and above [28].

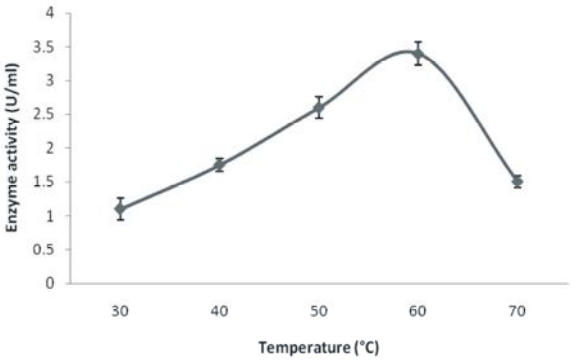

Fig. 2: Effect of temperature on protease activity. The result was the mean of three different repeats.

Effect of Divalent Ions on Enzyme Activity: The effect of divalent ions on the enzyme activity was assayed with divalent ions (5 mM) namely, Ca^{2+}, Mg^{2+}, Hg^{2+}, Zn^{2+} and Cu^{2+}. Among the divalent ions Ca^{2+} ion enhanced enzyme activity with a relative enzyme activity of 120%. Relative enzyme activity had decreased to 72, 86, 80 and 20% in copper, magnesium, zinc and mercury, respectively (Fig. 3). These results were in accordance the result obtained with *Homarus americanus*, in which Hg^{2+} and Cu^{2+} strongly inhibited protease activity and Ca^{2+}, Mg^{2+} had little or no inhibition of proteinase activity [14].

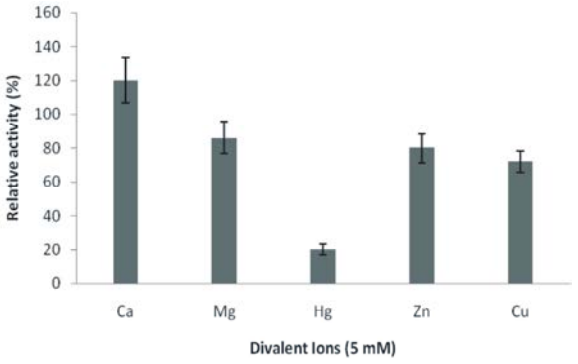

Fig. 3: Effect of divalent ions on protease activity. The result was the mean of three different repeats.

Effect of Activators/inhibitors on Protease Activity: The catalytic properties of the enzyme was studied with 5 and 10 mM serine protease inhibitor phenyl methyl sulphonyl floride (PMSF), it showed little or no effect on enzyme activity. The relative enzyme activity was 100% at 5 PMSF and 97.5% at 10 mM. Presently study revealed that this protease belongs cysteine proteases because it was widely active in the presence β-mercaptoethanol and Dithiothreitol (DTT) at both 5 and 10 mM concentration. Enzyme activity increased 6 and 9 fold in the presence of 5 and 10 mM β-mercaptoethanol. The dithiothreitol enhanced 3 and 8 fold protease activity. These results are

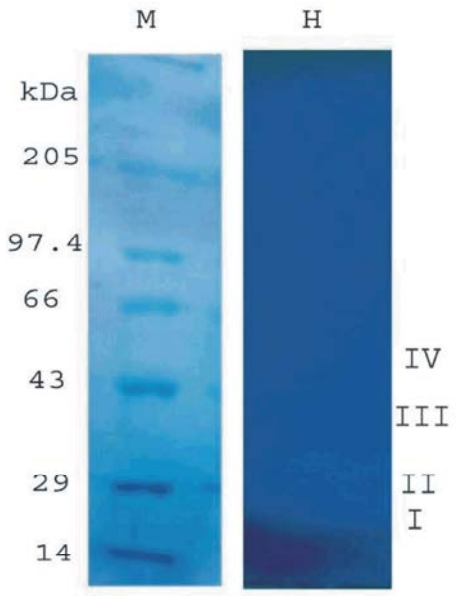

Fig. 4: Zymography of protease from the Lobster, *Panulirus homarus* (M: Protein molecular weight marker, H: Purified protease from hepatopancreas).

in good agreement with the result obtained by Laycock *et al*. [14]. The activation of lobster enzyme by β-mercaptoethanol and dithiothreitol are characteristic of cysteine proteinases [14].

Purification and Zymogram of Cysteine Protease from the Lobster *Panulirus homarus*: The cysteine protease was purified by the combination of ammonium sulphate precipitation, ion exchange chromatography and gel filtration. Final purification yielded 8.3 fold with 32.5% recovery. Similar two steps chromatographic procedure was carried out earlier by Galgani and Nagayama [13] with *Panulirus japanicus*. In this present study zymogram showed four activity bands on SDS-PAGE and the molecular weight was 27, 31.5, 38.4 and 45.5 kDa for bands I, II, III and IV, respectively. Among the four bands, I and II were l ikely to be cysteine protease, because β-mercaptothanol strongly activated these two proteases (Fig. 4). Among Lobsters, the protease activity bands were determined on SDS-PAGE at different range of molecular weight. Laycock *et al.* [14] evidenced at least six protease bands on SDS/PAGE from *Homarus americanus*. But in Norwegian Lobster *Nephrops norvegicus*, three proteases were identified in SDS-PAGE. Among three (I, II and III), I and II were likely to be thiol proteases and the molecular weight was found to be 22.5, 45 and 42.5 kDa [29] and Sun and Lopata [30] reported five major protease active bands at the range from 10 kDa to 20 kDa in rock lobster.

It was concluded that that the cysteine protease isolated from the lobster, *P. homarus* digestive gland is thermostable. In fish processing plants, this mid-gut gland is considered as a waste and this can be exploited for the production of proteases for various biotechnological applications. This kind of study may be a lime-light for recycling the lobster mid-gut waste in a profitable manner.

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