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Heat-stable Alkaline Proteinase from Shrimp Waste

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Proteolytic activity of shrimp waste at alkaline pH was examined. Shrimp waste was found to be a good source for this enzyme. The enzyme activity in the shrimp waste was about 50 times higher than that of muscle. The pH optimum of the enzyme was 8.5 and temperature optima, 50-55°C. It was inhibited maximally by serine protease inhibitor such as STI (soybean trypsin inhibitor). An interesting feature of the enzyme was its tolerance to different solvents.

Key words: Shrimp waste, alkaline proteinase, solvent-tolerance

Alkaline proteinase is one of the major industrially important enzymes. The estimated world sales of this enzyme is US $ 650 million (Thomas, 1996). The enzyme has become a standard ingredient in premium detergents and also finds application in leather industry for soaking, bating and dehairing of skins and hides. The enzyme has been used for extraction of quality silk from silk worm. To-date, alkaline proteinases are produced commercially by bacterial fermentation (Godfrey, 1983). Majority of bacteria secrete meagre amounts of enzymes and thus cannot be employed for its large scale production. It is very difficult, time consuming and costly to isolate and develop bacterial strains suitable for production of enzymes. Therefore, the possibility of using shrimp waste as an alternative source of this enzyme was explored. The shrimp industry produces a large amount of waste. In India, about 60000 tonnes of this waste are generated annually (Udgate & Khuntia, 1994). Its quantum is further increasing every year due to spurt in crustacean aquaculture. The processing of shrimp waste for enzymes may add good value to the otherwise low value material. Very high alkaline proteinase activity was observed in shrimp waste. In this paper, the isolation and characterization of alkaline proteinase from shrimp waste are reported and its potential as a source for commercial production of the enzyme is discussed.

Materials and Methods

Shrimp (*Penaeus indicus*) waste from a commercial shrimp processing plant was brought to the laboratory in ice. A 10% homogenate of this waste (w/v) was prepared with 10% KCl solution (w/v) by stirring for 30 min with an overhead stirrer at room temperature. The homogenate was filtered and centrifuged at 12,000 x g for 20 min in refrigerated centrifuge (Sorvall RC-2) at 0-4°C. The supernatant was filtered through muslin cloth.

The filtrate was incubated at 50°C for 30 min and cooled in ice. This was centrifuged at 12,000 x g for 20 min in refrigerated centrifuge at 0-4°C. The supernatant was collected and used for enzyme assays.

The azocaseinolytric activity of the extracts was determined using azocasein as substrate in a 2.0 ml reaction system containing 0.6% azocasein, an aliquot of enzyme extract and borate buffer (0.1 M, pH 8.5).

After incubation at 55°C for 10 min, reaction was terminated by adding 2 ml of 10% trichloro acetic acid (TCA) and the absorbance of TCA filtrate was measured at 366 nm (Sherekar *et al.*, 1997). A unit of activity corresponds to change in absorbance by 0.001 per min.

Caseinolytic activity was determined using casein as substrate in a 2.0 ml reaction system containing 1.0% casein, an aliquot of enzyme extract and 0.1 M borate buffer (pH 8.5). After incubation at 55°C for 10 min, reaction was terminated by adding 2 ml of 10% TCA. Tyrosine in the TCA filtrate was estimated by Miller's method (Miller, 1959). Activity was expressed in tyrosine equivalents.

The protein concentration was determined by Miller's method (Miller, 1959) with bovine serum albumin as a standard.

The enzyme activity in the pH range of 4.0-10.0 was determined. The azocasein substrate was prepared in different buffers such as 0.1 M acetate buffer (pH 4.0-5.0), phosphate buffer (pH 6.0-7.5), 0.1 M borate buffer (pH 8.0-9.0) and 0.1 M glycine/NaOH buffer (pH 10.00). The enzyme activity was determined using azocasein as described above. Stability of the enzyme at different pH was determined by incubating aliquots (0.1 ml) of enzyme with 1.9 ml of buffers of different pH (Table 4) for 3 h at 0-4°C. The residual enzyme activity was then determined.

The optimum temperature for the enzyme was determined by carrying out the assay at various temperatures (25-60°C). Temperature stability was determined by incubating aliquot of the enzyme at different temperatures (25-70°C) for 30 min and determining the residual activity.

The effect of inhibitor on the enzyme activity was studied by incubating the enzyme with inhibitors (Table 5) at 25°C for 30 min and then determining the residual activity. Inhibition of the enzyme activity by various solvents was also studied. The enzyme was incubated with different solvents (Table 6) in the ratio of 3:1 in stoppered tubes at 25°C with continuous shaking in a rotary shaker. The residual enzyme activity was determined at various intervals.

Results and Discussion

Table 1 shows the proteolytic activities of shrimp waste. Caseinolytic and azocaseinolytic activities of shrimp waste were 582.3 and 140.3 units respectively. The enzyme activity of prawn waste extract was much higher when compared with other prawn varieties reported earlier (Sherekar *et al.*, 1997). The significant difference in proteolytic activity is related to the raw materials used in the preparation of extracts. The muscle of prawn and Jawala as whole were used in the preparation of extracts in earlier studies. In the present studies, shrimp waste consisting of mainly head region was used for extraction of the enzyme. Shrimp being a crustacean, the hepatopancreas is located in the cephalothorax region of the head and this organ has been shown to be a good source of enzymes (Olsen *et al.*, 1991; Sherekar *et al.*, 1997). Very high activity associated with shrimp waste indicates that it can be used as a raw material for the large scale extraction of the enzyme.

Table 1. Proteinase activity in shrimp waste at pH 8.5

Substrate	Specific activity
Casein	582.3±52.1*
Azocasein	140.3±16.3@

* nmole tyrosin/mg/min;
@ units/mg/min

The degree of extraction of the enzyme with different concentrations of potassium chloride solutions was examined (Table 2).

The extractability of the enzyme increased with increasing concentration of the salt solution.

Table 2. Extraction of alkaline proteinase with different concentrations of KCl

Concentration of KCl (%)	Azocaseinolytic specific activity*
1.0	138.9
1.5	159.0
2.0	162.4
3.0	181.3
5.0	268.9
10.0	284.8

*Units/mg/min; average of three experiments

The ratio between shrimp waste and extractant (w/v) was varied and the degree of extraction of the enzyme assessed. The data given in Table 3 show that the extractability of the enzyme was slightly more with 10% homogenate when compared to 50% homogenate. However, preparation of 50% homogenate was considered a better option for large scale process, keeping in mind the amount of raw materials to be processed. Further, 10% extract would involve large quantities of liquid for downstream processing.

Table 3. Effect of the ratio between KCl solution (extractant) and shrimp waste on the alkaline proteinase activity of the extract

Waste:extractant (wt/vol)	Specific activity (for casein) (nmole tyrosine/mg/min)
10	526.06
30	522.92
50	498.70

The pH optimum of the enzyme was determined at 55°C using azocasein as substrate. The results showed that the hydrolysis of azocasein was spread over the pH range of 7-10 with maximum activity at pH 8.5 (Fig.1). Rapid loss of activity was

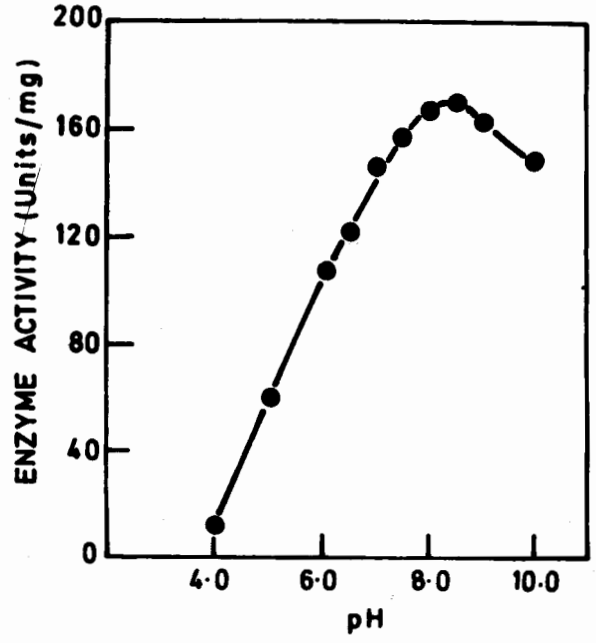

Fig. 1. Effect of pH on the activity of shrimp waste alkaline protease.

observed below pH 6.0. The enzyme was incubated at various pH (4.0 to 10.0) for 3 h and found to be quite stable (Table 4). Similar alkaline proteinases have been reported from shrimp muscle (Doke & Ninjoor, 1987) and Jawala prawn (Sherekar *et al.*, 1997).

Table 4. Effect of pH on the stability of alkaline proteinase from shrimp waste

pH	Azocaseinolytic specific activity* (units/mg/min)
4.0	146.40
5.0	146.97
6.0	160.80
7.0	163.97
8.0	152.73
9.0	146.39
10.0	144.66

*average of three experiments

The effect of temperature on enzyme activity using azocasein as substrate (pH 8.5) is shown in Fig.2. The enzyme showed maximum activity at 50-55°C. An important feature about the enzyme extract was its

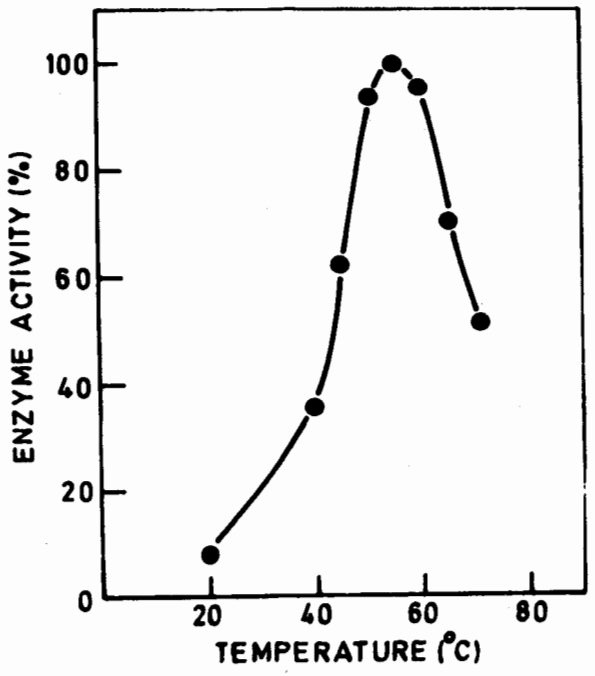

Fig. 2. Effect of temperature on the activity of shrimp waste alkaline protease.

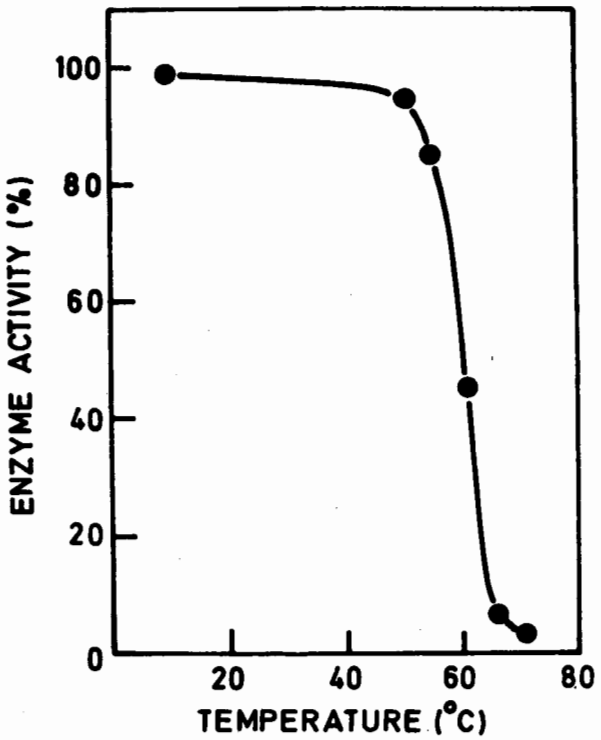

Fig. 3. Effect of temperature on the stability of shrimp waste alkaline protease.

thermal stability. The enzyme retained 95% of its activity over a period of half an hour at 50°C while 80% activity was retained at 55°C. Incubation at temperatures above 65°C resulted in inactivation (Fig.3). Shrimp waste enzyme resembles alkaline proteinases from other marine sources (Makinodan *et al.*, 1985).

Table 5. Effect of inhibitors on the activity of alkaline proteinase from shrimp waste (substrate-azocasein)

	Final concentration	% residual activity	(%) inhibition
None		100	0
STI	20 µg/ml	22.4	77.6
Leupeptin	10 µM	54.9	45.1
EDTA	5 mM	55.8	44.2
Benzamidine	2 mM	70.0	30.0
PMSF	1 mM	65.5	34.5
Chymostatin	20 µg/ml	70.5	29.5
TLCK	50µM	81.4	18.6
Pepstatin	15µM	100	0
E-64	10µM	100	0

The influence of inhibitors on the activity of the enzyme is presented in Table 5. Alkaline proteinase was strongly inhibited by trypsin inhibitor, STI and inhibition was 77.6%. EDTA and leupeptin caused about 45% inhibition. Pepstatin and E-64 did not affect the enzyme. Inhibition by STI and PMSF indicates that the enzyme may be a serine type proteinase and inhibition by EDTA suggests that the enzyme is metal

Table 6. Effect of solvents on the activity of alkaline proteinase from shrimp waste (Substrate-azocasein)

Solvent	Enzyme activity (Units/mg) 1 h	6 h	24 h
None	125.92	125.96	138.04
Hexane	134.0	133.33	169.36
Petroleum ether (60-80°C)	107.74	109.76	160.60
Toluene	115.82	139.39	170.03
Cyclohexane	121.88	144.44	184.17
Xylene	118.51	132.6	182.49

dependent. Further, these studies showed the presence of more than one proteinase, as evidenced by the absence of complete inhibition with any one inhibitor and partial inhibition with many. Serine proteinases have been demonstrated in muscle of white prawns (Doke & Ninjoor, 1987) and *Acetes indicus* (Sherekar *et al.*, 1997).

The data on the effect of different solvents on the enzyme activity (Table 6) show that the enzyme is stable in the presence of various solvents and no change in activity was observed over a period of 24 h. This is an important feature of the enzyme because of the current interest in enzymatic reactions using proteinases in the presence of organic solvents for the synthesis of peptides and esters (Tai *et al.*, 1989; Barros *et al.*, 1992). In organic solvents, the reaction equilibrium of proteinases can be shifted towards completion of synthetic reaction rather than hydrolysis. Therefore, proteinases are increasingly being used in nonaqueous media to enhance the solubility of substrates or products and for peptide synthesis which are thermodynamically unfavourable in water (Cassells & Halling, 1989). Proteolytic enzyme which is naturally stable in organic solvents and secreted by a bacterium tolerant to these solvents has been reported (Ogino *et al.*, 1994). Shrimp enzyme is another example of solvent stable enzyme.

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