ISSN: 0975-833X

*Available online at http://www.journalcra.com*

*International Journal of Current Research*
*Vol.3, Issue, 6, pp.036-041, June, 2011*

**INTERNATIONAL JOURNAL OF CURRENT RESEARCH**

**RESEARCH ARTICLE**

# ISOLATION AND CHARACTERIZATION OF PROTEASES FROM THE VISCERA OF *Labeo rohita*

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**ARTICLE INFO**

***Article History:***
Received 9th March, 2011
Received in revised form
7th April, 2011
Accepted 5th May, 2011
Published online 2nd June 2011

***Key words:***
*Labeo rohita*,
Fish viscera,
Zymogram,
SDS-PAGE,
PMSF,
Protease applications.

**ABSTRACT**

A preliminary study on the isolation and characterization of proteases from the crude extract of visceral organs of *Labeo rohita* were carried out. In order to estimate the number of proteases and their mass, the crude protease extract were separated by SDS-PAGE and then the activity was revealed by Zymogram. The crude enzyme extract showed two clear bands on casein and gelatin Zymogram, which indicated the presence of two major proteases. The optimal pH and temperature of the proteases obtained in crude extract were 8.0 and $40^0$ C respectively, using casein as a substrate. Furthermore, the crude enzymes were characterized by protease inhibitors namely phenylmethylsulphonyl fluride (PMSF), EDTA and β mercaptoethanol, in which the protease enzymes were strongly inhibited by PMSF (serine protease inhibitor). In addition, the enzymes were found to be highly active in the presence of activators like $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$ and $Na^{+}$. The protease kinetic constants, $K_m$ and $V_{max}$ of the crude enzymes for casein, were 0.65 mM and 2.5 U/ml/min respectively. With respect to properties of the enzyme and its capacity for degradation of different protein sources, these proteases finds potential application for waste treatment used in detergent and leather industry.



## INTRODUCTION

Proteases, catalyzes the total hydrolysis of proteins (Sharmin *et al.*, 2007). They execute a large variety of functions and have important biotechnological applications. Proteases represent one of the three largest groups of industrial enzymes and find applications in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes (Anwar *et al.*, 1998 and Gupta *et al.*, 2002). Proteases are mainly derived from plant, animal and microbial sources, where as their counterparts are derived from marine and other aquatic sources that have not been extensively used (Haard *et al.*, 1994). During the processing of fish only fillets were retained while the bulk of products (up to 66%) were discarded. Some of these fish wastes were rendered, but most was dumped to landfills (Knuckey *et al.*, 2004). Therefore, there is a need to find ecologically acceptable means for reutilization of these wastes (Ennouali *et al*., 2006). However fishing by products is typically used as feeds and fertilizers that have low dollar value. There is a growing interest in obtaining higher value biochemical and pharmaceuticals from fishery wastes, notably enzymes (Haard, 1998). Hence a trial has been carried out to isolate and characterize the digestive proteases of *Labeo rohita* and further confirmation was made to detect protease activity with few applications.

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## MATERIALS AND METHODS

### Analytical reagents

Casein, bovine serum albumin (BSA), trichloroacetic acid (TCA), folin ciocalteu's reagent, sodium carbonate, Tris (hydroxylmethyl) aminomethane, Coomassie Brilliant Blue R-250, acrylamide, bisacrylamide, N, N, N', N', tetramethyl ethylene diamine (TEMED), sodium dodecyl sulphate (SDS), ammonium persulfate and ethylene diamine tetraacetic acid (EDTA), phenylmethylsulfonyl fluride (PMSF), β-mercaptoethanol and glycine were purchased from Sigma Chemicals, (USA). Protein standard markers (14.4-116.0 KDa) were purchased from Bio-Rad (Mississauga, Ontario, Canada). Dialysis tubing was purchased from Amershan Phamacia Biotech (Uppsala, Sweden). All other reagents were of analytical grade.

### *Labeo rohita* viscera

*Labeo rohita* were purchased from the local market in Mettur Dam, Tamilnadu, India. The fish were kept in ice and transported to the research laboratory within 1 hr. After washing the fish with distilled water, visceral organs were separated and used immediately for the extraction of digestive enzymes.

**Preparation of crude protease extract**

Viscera from *Labeo rohita* (80 g of fish intestine and pyloric caeca) were weighed and homogenized with 250 ml of 10 mM Tris HCL buffer (pH 8.0) (Barkia *et al.,* 2009). The homogenate was centrifuged at 8500 × g for 30 min at $4^0$ C. The pellet was discarded and the supernatant was collected and used as crude protease extract.

**Assay of protease activity**

Protease activity was assayed by Anson method (1938) with some modifications. The enzyme solution (0.5 ml) was mixed with 5.0 ml of substrate (0.65% casein in 25 mM Tris HCL buffer, pH 8.0) at room temperature for 30 minutes and after incubation, TCA (110 mM) was added to attenuate the reaction. This mixture was incubated for 30 minutes at room temperature and centrifuged at 10,000 × g at $4^0$ C for 15 minutes, Release of amino acids were measured as tyrosine by the method of Folin and Ciocalteu (1929).

**Protein Determination**

Protein concentration was estimated by Lowry *et al.,* (1951) using Bovine Serum Albumin as standard.

**SDS-PAGE**

Sodium Dodecyl Sulphate (SDS)-PAGE was performed to determine the molecular weight of crude enzyme using the method of Laemmli (1970), using 4% stacking gel and 10% (W/V) separating gel. The samples were prepared by mixing the crude enzyme extract with distilled water containing 0.062 M Tris, 4% SDS, 10% glycerol, 1.5% β-mercaptoethanol and 0.002% bromophenol blue (pH 6.8). The polyacryamide (monomer 8.33% concentration) vertical gel tubes containing 0.15% SDS and 0.375 M Tris, 6.1 g glycine and 0.25 g SDS per 250 ml. Then 10 µl of sample was applied on the gel surface and fractionated for 4 hours at 50 volt. The gel was stained at half an hour (methanol-50 ml, acetic acid -7 ml, CBB R 250-250 mg, distilled water to make up to 100 ml). Then the gel was destained using distilled water, methanol and acetic acid in the ratio of 50:7:43. Standard proteins (Bio-Rad) used for calibration were β galactosidase (116 kda), Bovine serum albumin (66.2 kda), ovalbumin (45 kda), Lactate dehydrogenase (35 kda), REase BSP981 (25 kda), β-lactoglobulin (18.4 kda), Lysozyme (14.4 kda).

**Detection of protease activity by Zymogram**

Proteases obtained from *Labeo rohita* viscera were resolved in 7.5% sodium dodecyl sulphate (SDS)-polyacrylamide gels containing the following substrates: 0.1% gelatin, and 0.1% casein. Samples (10 µl) were mixed with sample buffer (10 mM HCL) and loaded. The protein sample was electrophoresed under standard conditions. After electrophoresis, SDS was removed from the gel by immersing in a solution containing 2.5% Triton X 100 and then the gels were incubated overnight at room temperature in 50 mM Tris Hcl (pH-8.0) buffer supplemented with $CaCl_2$ (1 mM), $ZnCl_2$ (0.001 mM) and NaCl (150 mM). The protease activity was visualized by staining the gels by Coomassie brilliant blue (Twinig *et al*., 1993).

**Characterization of Crude protease**

**Effect of pH on protease activity**

Protease activity was assayed over the pH range of 4.0-12.0 at $40^o$ C for 30 min using casein as a substrate. The optimum pH of the protease enzyme was determined by preparing the casein substrate in various buffer solutions (100mM Sodium acetate buffer, pH4.0-6.0; 100 mM Phosphate buffer, pH 7.0; 100 mM Tris HCL buffer, pH 8.0-9.0; 100 mM Glycine-NaoH buffer, pH 10.0-12.0.

**Effect of Temperature on protease activity**

To investigate the effect of temperature, on crude protease extract, the reaction mixtures were tested at different temperatures ranging from 20 to $80^o$ C, using casein as a substrate for 30 min at pH 8.0.

**Effect of inhibitors on protease activity**

The effect of enzyme inhibitors on the activity of protease were studied using phenylmethyl sulphonyl fluride (PMSF), β-mercaptoethanol and ethylene diamine tetraacetic acid (EDTA). The crude extracts were preincubated with each inhibitor for 60 min at 40º C and then the remaining enzyme activity was tested using casein as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as control.

**Effect of activators**

The effect of various metal ions (5mM) on crude enzyme activity were determined using casein as a substrate by adding monovalent and divalent metal ions ($Alcl_3$, $Bacl_2$, $Cacl_2$, $CdSo_4$, $CuSo_4$, $Hgcl_2$, KCL, $Licl_2$, $Mgcl_2$, $Mncl_2$, $Cocl_2$ and $ZnSo_4$) to the reaction mixture. The activities of the enzyme assayed in the absence of metal ions were taken as control.

**Assay of Kinetic parameters of enzyme**

The activity of the crude protease was evaluated at 40ºC with different final concentrations of casein ranging from 6.5 mg to 45.5 mg. The final enzyme concentration for the assay was 32.5 mg/5 ml. The respective kinetic parameters, including the Michaelis – Menten constant, $K_m$ and maximum velocity, Vmax were evaluated by plotting the data on a Line weaver-Burk double reciprocal graph (Line weaver and Burk, 1934).

**Enzyme applications**

**Dehairing of goat skin**

Goat skin was cut into 4 x 3 cm pieces and incubated with the crude protease (2.06 U/ml) in 10 mM Tris HCL buffer pH 8.0 at $40^o$ C. The goat skin was checked for dehairing at different incubation times.

**Destining property of enzyme (Ramakrishna *et al*., 2010)**

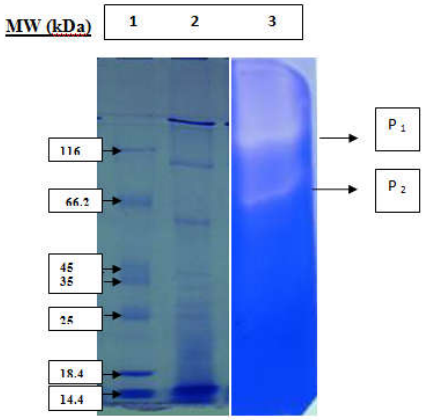
A clean piece of white cloth (5 x 5) was stained with soya sauce and allowed to dry. The cloth was soaked with 2% formaldehyde for 30 min and washed with water to remove the

excess of formaldehyde. Then the cloth was incubated with the crude proteases (2.06 U/ml) and 0.5 g of Ariel detergent at 40° C incubation. After incubation, cloth was rinsed with water for 2 min and then dried. The same procedure was performed for the control, except incubation with the enzyme solution.

## RESULT AND DISCUSSIONS

In the present study the crude protease enzyme was analyzed by SDS-PAGE and Zymogram and showed the presence of two caseinolytic proteases with the molecular weight of 90 and 55 Kda (Fig. 1).

**Figure 1**




### Effect of pH on protease activity

The pH activity profile of the crude Rohu protease extract was shown in Fig 2. The effect of pH on the activity of the crude protease was studied using casein as a substrate at a pH range of 4.0-12.0 at 40° C. The activity of the crude protease increased with increase in pH and reached its maximum at pH 8.0. However protease activity decreased significantly above 11 and was only 37 % of activity was observed at pH 12.0. El-Beltagy *et al.,* (2005) observed that Optimum pH for alkaline protease extracted from viscera of bolti fish (*Tilapia nilotica*) was 8.0 and similar results were obtained for Hayet (2011) in *Sardinella aurita*.

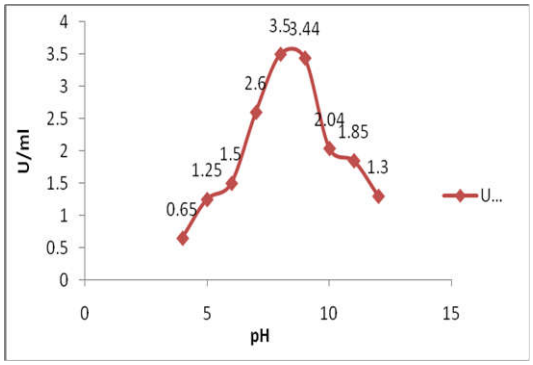



**Fig. 2: Optimum hide the value of Crude extract of *Labeo rohita***

### Effect of Temperature on protease activity

The effect of temperature on crude protease activity was determined by assaying at different temperatures. Fig. 3 shows that protease from Rohu was active at temperatures from 30 to 50° C with an optimum around 40° C, while slight activity was detected when assayed at 80° C. These data's were in good agreement with those reported by El-Beltagy *et al*, 2005.

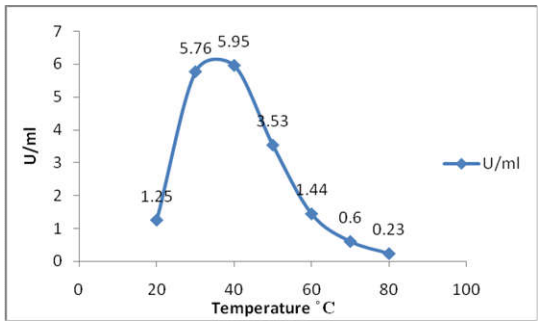



**Fig. 3: Optimum hide the value of Crude extract of *Labeo rohita***

### Effect of Inhibitors on activity

Proteases can be classified by their sensitivity to various inhibitors (North, 1982). In order to determine the nature of protease, the effects of different enzyme inhibitors were investigated. It was found that proteases in crude extracts were strongly inhibited by PMSF. The percentage of inhibition was found to be 73.4 and 90.6%, when a 1 and 10 mM concentrations were used (Table 1). These data's indicated that the proteases of *Labeo rohita* belonged to the serine proteases family. Similar results were observed with trypsin from other fish species namely *Skipjack* tuna spleen (Klomklao *et al.*, 2007), Yellowfin tuna (*T. albacores*), (Klomklao *et al.*, 2006), and Sardine (*S. pilchardus*) (Bougatef *et al.*, 2007). Further, the enzyme activity was highly affected by EDTA and β-mercapto ethanol which showed the residual activity of 43.43 and 87% in 10 mM concentrations.

**Table 1. Effects selected enzyme inhibitors on the activity of the crude *Labeo rohita* protease**

| Chemicals | Class | Concentraions (mM) | Residual activity (%) |
|---|---|---|---|
| Control | - | - | 100 |
| PMSF | Serine protease inhibitor | 1 | 26.6 |
| | | 10 | 9.4 |
| EDTA | Metallo protease inhibitor | 1 | 77 |
| | | 10 | 43.43 |
| β - mercapto ethanol | Cysteine protease inhibitor | 1 | 100 |
| | | 10 | 86 |

Activity was measured at pH 8.0 and 40° C with casein as substrate.

### Effect of Metal ions on activity

The effects of some selected metal ions at a concentration of 5mM were studied on the *Labeo rohita* crude protease extract at pH 8.0 and 40° C (Figure 4). Interestingly, the crude enzyme was found to be highly active in the presence of $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$ and $Na^{+}$. In addition, the presence of 5mM Kcl did not affected the protease activity. However, $Zn^{2+}$, $Ba^{2+}$, $Hg^{2+}$ and $Co^{2+}$ could inhibit the activity, particularly among them $Hg^{2+}$ and $Co^{2+}$ severely inhibited the enzyme activity. $Ca^{2+}$ highly increased the protease activity. Similar results were observed by purification of trypsin in other fish species namely *Sadinella aurita* (Khaled, 2011), *Pseudupeneus maculatus*

(Soza, 2007), *Sardina pilchardus*, (Bougatef, 2007) and *Sepia officinalis* (Balti, 2009). Inhibition of enzyme activity by $Hg^{2+}$ was also demonstrated in Bogue viscera (Ahmed Barkia *et al.*, 2010) and Hybrid *Tilapia* intestine (Wang *et al.*, 2010).

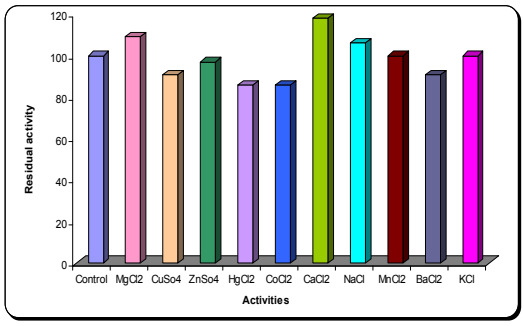



**Fig. 4: Effect of various metal ions on the activity of protease in crude extract**

## Kinetic studies

Kinetic constants $K_m$ and $V_{max}$ of the crude proteases were calculated using Michaelis-Menten and Lineweaver-Burk double reciprocal graph (Fig.5&6). The values of Km and $V_{max}$ of the crude enzyme using casein were 0.65 mg/ml and 2.5 U/min/ml respectively.

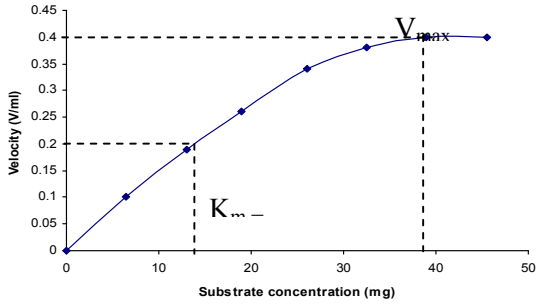



**Fig. 5. Michealis - Menten Kinetics**

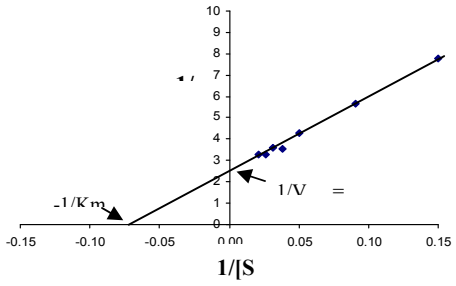



**Fig. 6. Line Weavever and Burk Plot**

## Dehairing of goat skin

Incubation of the protease with goat skin for dehairing showed that, after 5 - 6 hrs of the enzyme (2.06 U/ml) incubation with goat skin, hair was removed completely compared to the control (Plate 1). Protease isolated from the *Pseudomonas aeruginosa* has the ability of dehairing of cow skin (Najafi, *et al.*, 2005). Similarly protease from *Bacillus subtilis* has the potential application of dehairing of goat skin (Changediya *et al.*, 2010).

1. Control

2. After incubating the goat skin with protease from the visceral waste of *Labeo rohita.*

**Plate 1: Dehairing of goat skin with protease enzyme isolated from digestive tract of *Labeo rohita***

## Effects of proteases on stain removal

The efficiency of proteases isolated from *Labeo rohita* viscera were tested for the removal of stain, soya sauce from the fabric (cotton) at 40º C with 2.06 U/ml enzymes and 1% detergent. It took 2 hrs for the complete removal of stain at 40º C. But in the detergent alone (absence of enzyme), the time taken to remove stain was very long (Plate 2). It was reported that proteases from *Bacillus* sp.APR-*4* removed protein stains (egg yolk) with in 30 min in 100 U/ml enzymes at pH 9.0 (Kumar *et al*., 2004).

**STAIN (SOYA SAUCE)**


**Plate 2: Destaining of cotton cloth with protease enzyme isolated from digestive tract of *Labeo rohita***

**Conclusion**

In conclusion, the enzyme was similar in many biochemical characteristics to the proteases of other fish, and these characteristics suggest that the enzyme possibly could be an important biotechnological tool for the fish processing and food industries, there by contributing to the reduction of waste disposal problems.

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