Isolation and purification of protease from *Labeo rohita* viscera

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The discarding of fish wastes creates environmental and disposal problems. A number of bioactive compounds can be extracted and purified from the fish wastes. Therefore, protease was purified from the visceral organ wastes of *Labeo rohita*. The fish visceral waste was collected from Mettur Dam, Tamil Nadu. A crude homogenate was prepared and subjected to acetone precipitation, dialysis, Sephadex G-100 and DEAE cellulose column chromatography. The molecular weight of the isolated protease was determined by SDS-PAGE and confirmation of the protease activity was done by zymography. From the study, it was concluded that, the final purified sample showed a purification fold of 13.4, the specific activity of 8.62 U/mg, recovery percentage of 5.44 and a molecular weight of 38 kDa. Hence the present study was taken up with the view of utilizing the large amounts of fish wastes for the extraction of a beneficial enzyme, namely protease thereby contributing to the reduction of pollution caused by disposal of fish waste.

**Keyword:** Acetone, Crude enzyme, Digestive protease, Fresh water fish, Rohu, Visceral protease

Aquaculture has grown tremendously and has become an economically important industry in the world¹. Today, it is the fastest growing food-producing sector with the greatest potential to meet the growing demands of aquatic food²,³. India is the third largest producer of fish and the second largest producer of freshwater fish in the world⁴. The fish consumption per person has doubled on a worldwide basis and hence the fishery waste on land has also increased. The discarding of fish waste creates environmental problems as well as disposal problems. Therefore, there is a need to find ecologically acceptable means for reutilization of these wastes⁵,⁶. Studies have reported that a number of bioactive compounds can be extracted and purified from fish waste such as bioactive peptides, oligosaccharides, fatty acids, enzymes, water-soluble minerals and biopolymers⁷. Proteases have diverse applications in a wide variety of industries such as detergent, food, pharmaceutical and leather industries, peptide synthesis and recovery of silver from used X-ray films. Proteases are mainly derived from animal, plant and microbial sources⁸. For marine animals, proteases are mainly produced by the digestive glands. The most important digestive enzymes are pepsin, secreted from gastric mucosa, trypsin and chymotrypsin secreted from the pancreas, pyloric caeca, and intestine. Like the proteinases from plants, animals, and microorganisms, digestive proteinases from marine animals are polyfunctional enzymes catalyzing the hydrolytic degradation of proteins⁹.

The present study aims at isolating proteases from the viscera of *Labeo rohita*, which is abundantly consumed by populations of Salem. *Labeo rohita*, commonly called Rohu, is a member of the family Cyprinidae within the order Cypriniformes¹⁰. It is a prime cultured and important staple freshwater fish generally found in rivers, ponds, and reservoirs¹¹. This fish species is readily available in all the local markets of Tamil Nadu, India. The viscera are a waste and are not used as a source of food and thus are rejected. This leads to a large accumulation of wastes in commercial places contributing to pollution of water and soil resources. Moreover, very few reports are available that justify the extraction of enzymes directly from waste products without involving any expansive infrastructure and with a low-cost approach. This study therefore, is an effort to minimize pollution caused due to the ignorant generation of such wastes and at the same time utilize them for the benefit of mankind.

**Materials and Methods**

**Reagents**

Bovine serum albumin, casein, Trichloroacetic acid, Folin ciocalteu’s reagent, sodium carbonate, Tris (hydroxymethyl) aminomethane, coomassie brilliant blue R-250, acrylamide, bisacrylamide, N,N,N',N'-tetramethyl ethylenediamine (TEMED), sodium

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dodecyl sulfate, ammonium persulfate, ethylene diamine tetraacetate acid (EDTA), glycine, sephadex G 100, DEAE cellulose, protein standard markers (14.4–116.0 kDa) and dialysis tubing were purchased from Himedia, Mumbai, India. All other reagents were of analytical grade.

*Labeo rohita* viscera

The viscera of fish *Labeo rohita* were collected from the local market in Mettur Dam, Tamil Nadu, India. The visceral samples were kept in ice and transported to the place of work within an hour. It was then washed with distilled water and stored in sealed plastic bags at −20°C.

**Preparation of crude enzyme extract**

The collected viscera were thawed for about 2 h at room temperature and then homogenized with 200 mL of homogenization buffer (10 mM Tris HCl, pH 8.0). The homogenate was centrifuged at 8500 × g for 30 min at 4°C. The pellet was discarded and the supernatant (50 mL) was collected and used as the crude protease extract.

**Acetone precipitation**

In the 50 mL of crude protease extract, an equal volume of ice-cold acetone (50 mL) was added in a dropwise manner with continuous stirring on ice. After the addition of the organic solvent is completed, the stirring was continued on ice for 10–20 min. The mixture was transferred to the chilled screw-cap polycarbonate centrifuge tubes and recovered the precipitated proteins by centrifugation at 10000 × g for 10 min at 4°C. The supernatants were discarded and the centrifuge tubes were inverted over filter paper for air drying. Then, the pellets were suspended in 15 mL of 25 mM Tris-HCl buffer (pH 8.0).

**Dialysis**

The precipitate obtained from the ice-cold acetone precipitation was dissolved in 25 mM Tris-HCl buffer at pH 8.0 and dialyzed against the same buffer for 24 h at 4°C. The buffer was changed twice (after 8 and 16 h).

**Sephadex G-100 column chromatography**

The dialysate was subjected to gel filtration on Sephadex G-100 column (2.0 cm × 50 cm) equilibrated with 25 mM Tris HCl buffer, pH 8.0. Each 5 mL fractions were eluted at a flow rate of 30 mL/h with the same buffer and analyzed for protein content and protease activity. Fractions showing the high protease activity were pooled and subjected to further purification steps.

**DEAE cellulose anion exchange column chromatography**

The active fractions pooled from Sephadex G 100 purification were applied to DEAE cellulose column (2.0 cm × 10 cm) pre-equilibrated with 25 mM Tris HCl, pH 8.0. The column was washed with the same buffer and then adsorbed proteins were eluted with a linear gradient of sodium chloride in the range of 0.1–1.0 M. Each 2 mL fraction was collected at the flow rate of 60 mL/h. Then, analyzed for protease activity and protein concentration. The fractions with high protease activity were pooled together and stored at −20°C for further characterization. All purification steps were conducted at temperatures not exceeding 4°C.

**Protease activity**

Protease activity was assayed by the Anson method with some modifications. The enzyme solution (1 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 min. After incubation, TCA (110 mM) was added to attenuate the reaction. This mixture was allowed to incubate for 30 min at room temperature and filtered to remove the precipitate. Then 2 mL of the filtrate was taken in a test tube and 1 mL of the Folin Ciocalteu’s reagent was added. The absorbance was measured at 660 nm. A standard curve was generated using solutions of 0.2 mg/mL tyrosine. One unit will hydrolyze casein to produce colour equivalent to 1.0 µmole (181 µg) of tyrosine per minute at pH 8.0 at 37°C.

**Protein determination**

Protein concentration was estimated by Lowry et al., using bovine serum albumin as standard.

**SDS-PAGE**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to determine the molecular weight of partially purified enzyme by the method of Laemmli using 4% stacking gel and 10% (W/V) separating gel. The samples were prepared by mixing the purified enzyme extract with distilled water containing 1M Tris, 20% SDS, 100% glycerol, 0.5 ml β-mercaptoethanol, and 0.01% bromophenol blue (pH 6.8). The polyacrylamide vertical gel tubes contained 10% SDS and 1.5 M Tris, 6.1 g glycine and 0.25 g SDS per 250 mL. Then, 10 µL (20 µg) of the sample was applied on the gel surface and fractionated for 4 h at 50 V. The gel was stained at half an hour (methanol, 50 mL; acetic acid,7 mL; CBG 250, 250 mg; distilled water to make up to 100 mL). Then, the gel was...
destained using distilled water, methanol, acetic acid, and water in the ratio of 50:7:43. Standard protein markers used for calibration were β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase BSP981 (25.0 kDa), β-lactoglobulin (18. kDa), and lysozyme (14.4 kDa).

Detection of protease activity by zymography

Protease obtained from Rohu viscera was resolved in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels containing the following substrates: 0.1% gelatin, 0.1% casein. Samples (10 μL) were mixed with sample buffer (1M Tris, pH 6.8, 20% SDS, 100% Glycerol, 0.01% Bromophenol blue and Distilled water). Samples were not boiled. 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.$^{17}$

Results & Discussion

Purification of the L. rohita visceral protease

The fish visceral waste was collected and the crude homogenate was prepared. Then it was subjected to precipitation with acetone (1:1 ratio of solvent and sample) and dialysis. One part of the dialysate obtained was analyzed for purification profile and the other part subjected to further purification on Sephadex G-100 column. A total of 37 fractions (5 mL each) were eluted. The highest protease activity was recorded by fraction 11 followed by fraction 10 and 12. The fractions before 9 and after 12 recorded lower protease activities. Similarly, Figure 1 also shows the presence of three peaks in which the fractions 10, 11 and 12 form the highest.

The active fractions which exhibited the highest protease activity were then pooled together and loaded on the DEAE cellulose column and a total of 25 fractions were collected (2 mL each). The proteins were eluted using a linear gradient of sodium chloride. There was a gradual increase in the protease activity from fraction 3 to 7 then a gradual decrease. This increase may be due to the washing of the column and the subsequent elution of unbound proteins. However, on the treatment of the column

![Fig. 1 — Elution profile of Sephadex G 100 purified fish visceral protease](image1)

![Fig. 2 — Elution profile of DEAE cellulose purified fish visceral protease](image2)

![Fig. 3 — Plate 1-SDS PAGE of DEAE cellulose purified fish visceral protease](image3)
This observation indicates that maximum protease activity was exhibited in fractions 10 to 13 with the highest value for fraction 11. The active fraction which showed the highest protease activity was divided into two. One part of it was used to study the purification profile (protease activity, protein content, specific activity, recovery percentage and purification fold) and the other part used for characterization studies. A summary of the purification is presented in Table 1.

### Table 1 — Summary of the purification of protease from *Labeo rohita*

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total protease activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>103.00 ±5.26</td>
<td>160 ±9.47</td>
<td>0.64 ±0.21</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>Acetone precipitation</td>
<td>56.00 ±2.78</td>
<td>54.00 ±2.63</td>
<td>1.04 ±0.36</td>
<td>54.40 ±2.39</td>
<td>1.60 ±0.27</td>
</tr>
<tr>
<td>Dialysate</td>
<td>53.00 ±3.47</td>
<td>44.25 ±2.64</td>
<td>1.20 ±0.21</td>
<td>51.50 ±4.24</td>
<td>1.90 ±0.65</td>
</tr>
<tr>
<td>Sephadex G-100 filtrate</td>
<td>9.6 ±1.10</td>
<td>6.6 ±0.98</td>
<td>1.5 ±0.32</td>
<td>9.3 ±1.36</td>
<td>2.5 ±0.73</td>
</tr>
<tr>
<td>DEAE cellulose filtrate</td>
<td>5.60 ±1.58</td>
<td>0.65 ±0.11</td>
<td>8.62 ±1.43</td>
<td>5.44±0.94</td>
<td>13.40 ±2.58</td>
</tr>
</tbody>
</table>

[Results are the mean ± SD of triplicates; U - One unit hydrolyzes casein to produce colour equivalent to 1.0 μM (181 μg) of tyrosine per minute at pH 8.0 at 30°C]

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### Molecular weight determination and zymography

Plate 1 represents the SDS-polyacrylamide gel electrophoretogram of the DEAE cellulose purified protease. Lane 1 represents the standard markers with known molecular weights ranging from 14.4 to 116.0 kDa and Lane, 2 represents the DEAE cellulose purified sample. From the electrophoretogram, it can be seen that, there was a single clear band corresponding to a molecular weight of 38 kDa. This result confirmed the purified protease to be a homogenous one since it gave a single band.

The zymogram of the DEAE cellulose purified protease shows the presence of a single clear white band in the middle of the gel. This observation concludes that the presence of the band was due to the action of the protease on the substrate casein. The presence of a single clear white band also confirmed that the isolated protease did not have any isoenzyme.

### Conclusion

The present study was aimed at utilizing fish visceral waste as a potential source of the enzyme protease thereby trying to reduce the waste disposal problem. The visceral wastes were collected from *L. rohita*, purchased in Mettur Dam, Tamil Nadu, India. A crude homogenate was subjected to acetone precipitation, dialysis, Sephadex G-100 column and DEAE cellulose column chromatography. After the final purification step, the enzyme showed a purification fold of 13.4, the specific activity of 8.62 U/mg, recovery percentage of 5.44 and a molecular weight of 38 kDa.

### References

13. Geethanjali S & Subash A, Comparative Study on Precipitation Techniques for Protease Isolation and


