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# Partial Characterization of Protease from the Visceral Organ Waste of Cobia (*Rachycentron canadum*)

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**Abstract**
Proteases are the most important enzymes accounting for approximately 60% of the total industrial enzyme market. Its isolation from the visceral organ waste of Cobia showed the highest specific activity in the 40 – 50 % ammonium sulphate precipitated sample. Hence, that fraction was dialyzed and was found to have an increase in the specific activity from 2.0 – 3.2 U/mg. Purification of the dialyzed fractions on Sephadex G-100 column also revealed an increase in the specific activity from 3.2 – 3.5 U/mg. The molecular weight of the enzyme was found to be 32.0 KDa. Zymogram was done to confirm the presence of protease. The optimum pH and temperature of the isolated enzyme was shown to be 8 and 60˚C where the protease activity was found to be the highest.
**Keywords:** Cobia, alkaline protease, fish waste, enzyme

## 1. Introduction

Enzymes are delicate protein molecules necessary for life (Das and Prasad, 2010). They are molecules of relatively small size and are compact spherical structures that catalyze peptide bond cleavage in proteins (Aftab *et al*., 2006). Protease refers to a group of enzymes whose catalytic function is to hydrolyze (breakdown) proteins. They are also called proteolytic enzymes or proteinases. Proteases occur naturally in all organisms and constitute 1-5% of the gene content. Proteases are mainly derived from plant, animal and microbial sources, whereas, their counterparts are derived from marine and other aquatic sources that have not been extensively used (EL-Beltagy, *et al*., 2005).

Fish processing creates a large amount of wastes of high nutrient content which, if not properly processed for use in human or animal nutrition, is likely to be deposited in the environment creating pollution problems (Kotzamanis *et al*., 2001). The viscera are one of the most important wastes of the fish industry, for example the sardine industry and this product is recognized with high potential as a source of digestive enzyme. Proteases from fish viscera could be used in industrial applications, so the recovery of this kind of waste might be an alternative to the pollution problem that the fish industry produces (Castillo *et al*., 2004).

*Rachycentron canadum*, is commonly known as Cobia, Black kingfish (Kerala), Black salmon, Ling, Lemonfish, Crabeaters, Aruan tasek and Nei meen (Tamil). Cobia is pelagic and is normally solitary except for annual spawning aggregations. However, they will congregate at reefs, wrecks, harbours, buoys and other structural oases. They may also enter estuaries and mangroves in search of prey (http://en.wikipedia.org/wiki/Cobia).

## 2. Materials and Methods

### 2.1 Collection of Fish Visceral Organ Waste and Crude Preparation

Commonly sold fish species was surveyed in Coimbatore fish markets and the most widely consumed fish Nei meen (Cobia, *Rachycentron canadum*) was selected for the study. The visceral organ waste of the fish was collected soon after cutting, placed in clean plastic bags, maintained in ice and brought to the place of study. The waste was washed well with distilled water and stored at 4°C. It was then weighed, cut into small pieces and homogenized in 0.02M Tris HCl buffer, pH 7.8. The homogenate was then filtered through cheesecloth to obtain a clear solution.

### 2.2 Enzyme Purification

Ammonium sulphate precipitation is often used as the first purification and concentration procedure. It is based on the principle of salting out. Protease was precipitated from the crude homogenate at varying concentrations of ammonium sulphate ranging from 0-90%. The protease activity and the total protein content in each of the ammonium sulphate precipitated fractions were estimated and were used to calculate the specific activity of protease. The fraction with the highest specific activity was dialyzed to facilitate desalting of the proteins. The dialyzed fraction was further purified on a Sephadex G-100 column equilibrated with 0.02M Tris HCl buffer (pH 7.8). The fraction showing high protease activity was taken for the estimation of protein.

### 2.3 Determination of Molecular Weight of Protease

#### 2.3.1. SDS-Page

SDS-PAGE of the dialyzed fraction was carried out on a 10% gel.

 

**2.3.2. Zymogram**

Zymography is an electrophoretic technique based on SDS – PAGE that includes a substrate co-polymerized with polyacrylamide gel for the detection of enzyme activity.

**2.4 Characterization of Protease**

**2.4.1 Optimization of pH and temperature**

The optimum pH of the purified protease was determined by preparing the substrate casein in buffers of varying pH ranging from 6-12.

**2.4.2 Optimization of temperature**

The optimum temperature for the enzyme was determined by incubating the enzyme-substrate mixture at temperatures ranging from 20°C- 80°C followed by checking its activity.

## 3. Results

**3.1 Protease Activity, Protein Content and Specific Activity of Crude and Ammonium Sulphate Precipitated Samples of Cobia**

Ammonium sulphate precipitation was done in various range from 0-90%. The maximum protease activity and protein content was observed in the 40 – 50 % ammonium sulphate precipitated fraction (16.41 U/ml and 8.2 mg/ml respectively) when compared with the other fractions. The maximum specific activity was also seen in the 40 – 50 % precipitated fraction (2 U/mg) and hence this fraction was used for further purification.

**3.2 Protease Activity of Dialysate of Ammonium Sulphate Fractionated Fish Waste Samples**

The 40 – 50 % precipitated fraction which showed maximum specific activity was dialyzed and the protein content and specific activity was noted. The protein content of the 40 – 50 % precipitated fraction had decreased (8.2 to 6.0 mg/ml) after dialysis, but the specific activity had increased from 2.0 to 3.2 U/mg, which indicates the purification level.

**3.3 Elution Profile of Protease on Sephadex G-100 Column**

The dialysate of the fractioned sample was further purified on Sephadex G-100 column. The fractions 20-25, which showed the highest peak was pooled and taken for the estimation of protein which is shown in the figure 1. The specific activity was found to increase from 3.2 to 3.5 U/mg which is shown in the table I and the purification fold to be 1.6.

**3.4 Molecular Weight Determination of Protease**

The molecular weight of the enzyme protease was determined by SDS-PAGE.

**3.4.1 SDS-PAGE**

The precipitated sample, dialyzed sample and eluted sample were analyzed by SDS-PAGE to determine the molecular weight of the enzyme of the fish.

From the figure 2, it can be inferred that the molecular weight of the protease enzyme may be 32.0 KDa, when compared to the standard protein marker of 6.6 – 200 KDa. The thickness of the band shows the increase in the purity level of the protein.

**3.4.2 Confirmation of Presence of Protease by Zymography**

The proteolytic activity of the enzyme was confirmed on an activity gel/zymogram. The zymogram showed clear bands indicating the caseinolytic activity of the enzyme isolated from fish waste which is given in figure 3.

**3.5 Characterization of Protease**

The purified enzyme was partially characterized based on the pH and temperature.

**3.5.1 Determination of optimum pH**

The activity of the isolated and partially purified enzyme at various pH are recorded in figure 4 and it can be inferred from the figure that the optimum pH of the enzyme is 8, as the maximum protease activity (3.5 U/mg) was noticed at this pH. The enzyme also showed considerable activity at pH 9 and 10 (2.9 and 2.2 respectively). However the activity reduced drastically at pH 11. The enzyme can therefore be termed as an "Alkaline Protease".

**3.5.2 Determination of Optimum Temperature**

The optimum temperature of the isolated and partially purified enzyme was determined and the results are depicted in the figure 5. From the figure, it is clearly seen that the protease activity was highest at 60ºC (3.1 U/mg). There is also a considerable activity between 50 – 70ºC (2.5 and 2.6 U/mg respectively) beyond which the activity has decreased.

## 4. Discussion

From the findings of the study, it was seen that in Cobia, 40 – 50 % ammonium sulphate precipitated sample showed the highest specific activity (2.0 U/mg) and this fraction was taken for the further studies. The fraction was dialyzed and was found to have an increase in the specific activity from 2.0 to 3.2 U/mg.Purification

 

of the dialyzed fractions on Sephadex G-100 column also revealed increase in specific activity from 3.2 to 3.5 U/mg.

The molecular weight of the enzyme was found to be 32.0 KDa on SDS – PAGE. The zymogram, was done to confirm the presence of protease. The optimum pH and temperature of the isolated enzyme was shown to be 8 and 60˚C respectively, where the protease activity was found to be highest (3.5 U/mg and 3.1 U/mg respectively). From this, it can be concluded that the enzyme isolated is an alkaline protease.

Thus, from the present study it was shown that the huge amounts of fish waste generated, which causes pollution, can be used effectively for isolating industrially important enzymes like protease.

## 5. Fundings

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**Table I**
**Protease activity, protein content, specific activity and purification fold of crude, ammonium sulphate precipitated, dialyzed and Sephadex G-100 column purified samples of Cobia**

| S. No | Fish waste sample | Protease activity (U/ml) | Protein content (mg/ml) | Specific activity (U/mg) | Purification Fold |
|---|---|---|---|---|---|
| 1 | Crude | 19.75 | 9.2 | 2.2 | 0 |
| 2 | 40-50% ammonium sulphate precipitated fraction | 16.42 | 8.2 | 2.0 | 0.91 |
| 3 | Dialyzed 40-50% fraction | 19.14 | 6.0 | 3.2 | 1.5 |
| 4 | Sephadex G-100 column purified fraction | 19.45 | 5.5 | 3.5 | 1.6 |

 

**Figure 1**
**Elution profile of protease on Sephadex G-100 column of Cobia sample**

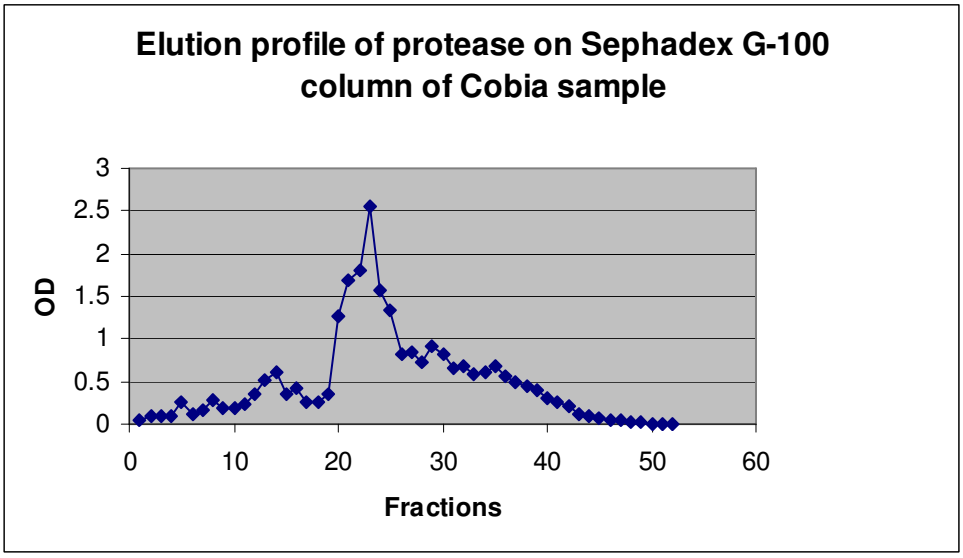


**Figure 2**
**SDS – PAGE of Protease from Cobia**

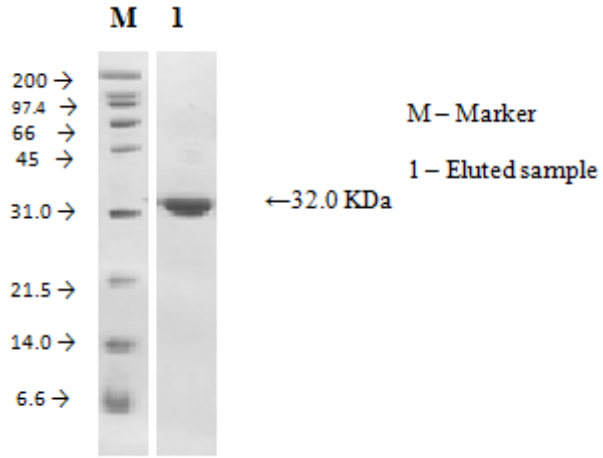

 

**Figure 3**
**Zymogram of Protease**

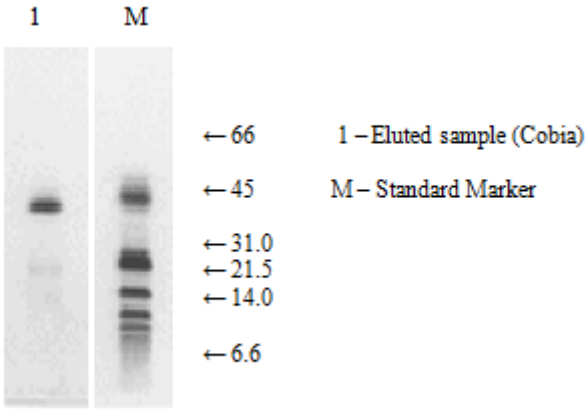


**Figure 4**
**Determination of optimum pH**

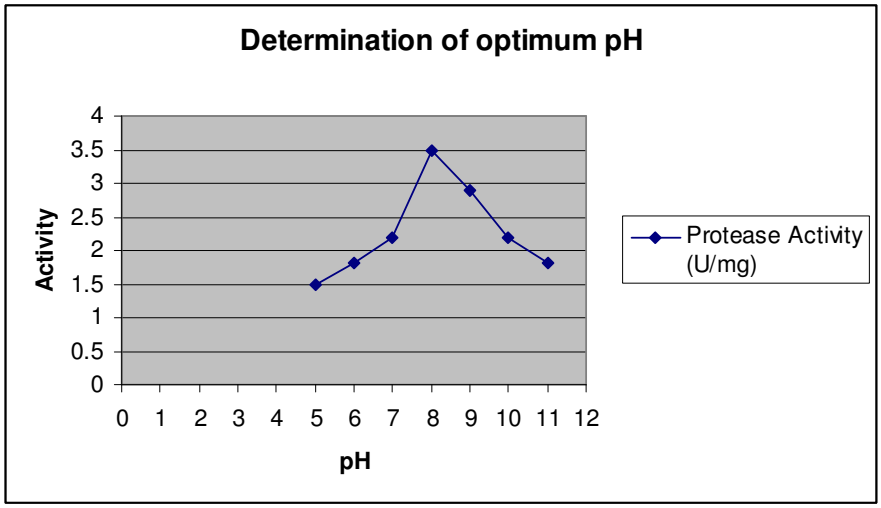


**Figure 5**
**Determination of optimum temperature**