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RESEARCH ARTICLE

A STUDY OF PURIFICATION PROFILE AND MOLECULAR WEIGHT OF FISH VISCERAL PROTEASE PRECIPITATED WITH DIFFERENT PRECIPITATING AGENTS

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ABSTRACT

The study was aimed at managing the vast amounts of wastes of the redfish *Lutjanus sebea* to isolate an industrially useful enzyme-protease. The visceral organ wastes of fish were collected, homogenized with Tris-HCl buffer and precipitated with varying concentrations of ammonium sulphate, acetone and ethanol and was then purified by dialysis and Sephadex G-100 column chromatography. The purification profile (the protein content, protease activity, specific activity, purification fold, and recovery %) were studied in the crude and partially purified samples. The results showed that the activity was maximum when precipitated with 40-60% ammonium sulphate, 100% acetone and 40% ethanol. The fractions with the highest specific activity were selected for further purification by dialysis and Sephadex G-100 column chromatography. The molecular weight of the isolated enzyme was determined by SDS-PAGE and Native PAGE and was to be found 27 kDa. The presence of protease was confirmed by zymography.

INTRODUCTION

Environmental pollution is a worldwide problem and its influence on the health of human beings is notable (Khan *et al.*, 2011). India is the third largest producer of fish and the second largest producer of fresh water fish in the world. Every year, 30 million tons of fish waste is dumped around the world and large amount of fish waste is transformed into a product that can be incorporated as ingredient in animal rations (Subash *et al.*, 2011). Enzymes are delicate protein molecules necessary for life (Das and Prasad, 2010). Proteases have wide applications in the biotechnology, research, food and detergent industries. The viscera are the most important wastes of the fish industry. Proteases from fish viscera are used in industrial applications and so the recovery of this kind of waste might be an alternative to the pollution problems (Castillo *et al.*, 2004). Today, there is an increasing demand for fish proteolytic enzymes in various industries. Utilization of viscera in proteolytic enzyme production can be a solution to meet this demand. Thus, in the present study, we focused on the isolation and partial purification of protease (enzyme that contribute to the application in various industries) from the viscera of Redfish (*Lutjanus sebea*).

MATERIALS AND METHODS

Analytical Reagents

Bovine serum albumin, casein, trichloroacetic acid, Folin-Ciocalteu reagent, sodium carbonate, tris (hydroxylmethyl) aminomethane, coomassie brilliant blue R-250, acrylamide, bisacrylamide, N,N,N',N'-tetramethyl ethylenediamine (TEMED), sodium dodecyl sulfate, ammonium persulfate, ethylene diamine tetraacetic acid (EDTA), and glycine were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Protein standard markers (14.4–116.0 kDa) were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Dialysis tubing was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents were of analytical grade.

Selection and Collection of Fish waste

The visceral organ waste of Redfish (*Lutjanus Sebea*) was collected from Coimbatore local market. They were kept at -20˚C soon after they were collected and brought to the laboratory. The samples were then used for enzyme extraction.

Preparation of Crude Homogenate

The fish waste was weighed and the tissues were cut into small pieces with a knife. These pieces were homogenized with 20 mM Tris-HCl buffer (pH 7.8) using a homogenizer. The homogenate was filtered using 8 layer cheesecloth. The homogenate was centrifuged at 10000 rpm for 15 min at 4˚C and the supernatant collected.

Precipitation of Protease by various precipitation methods
Ammonium sulphate precipitation

Protease was precipitated from the crude homogenate with saturated ammonium sulphate in varying concentrations from 0-100% (0-20%, 20-40%, 40-60%, 60-80%, 80-100%). The

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mixture was then centrifuged at 10,000 rpm for 20 minutes at 4°C and the pellet was collected.

Acetone precipitation

Acetone precipitation of protease was done with varying concentrations of ice-cold (-20˚C) acetone ranging from 10 – 100 % and the mixture was centrifuged at 13,000-15,000 rpm for 20 minutes at 4°C to get protease pellets from the crude.

Ethanol precipitation

Ethanol precipitation was done with varying concentrations of ice-cold (-20˚C) ethanol ranging from 10 – 100%. The ice-cold (-20˚C) ethanol was added to the crude supernatant and stirred slowly. The mixture was centrifuged at 10,000 rpm for 20 minutes at 4°C and the pellet was collected.

Dialysis

The protein fractions were desalted by dialysis using dialysis bags (membrane size 60) to prevent interference of salts with the protein function. Those ammonium sulphate, acetone and ethanol precipitated samples that gave the highest specific activity were dialysed against the same buffer (20mM Tris-HCl buffer pH 7.8) for 24 h at 4˚C with frequent changes after every 4 hours.

Purification of Protease by Gel filtration chromatography

The dialyzed fractions were further purified on Sephadex G-100 column of 1 × 20 cm. The column was equilibrated and eluted with 20 mM Tris-HCl buffer (pH 7.8). The elution was manually collected and the enzyme activity was measured in all the fractions using a spectrophotometer at 660nm. The fraction showing the highest peak was taken for the estimation of protein.

Assay of Protease

The enzyme activity was determined by the method of Anson, 1938. The enzyme solution (0.5 ml) was mixed with 5.0 ml 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˚C for 15 minutes. Release of the amino acid tyrosine was measured by the method of (Folin and Ciocalteu 1929).

Estimation of Protein content

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

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out with the crude extract, 40-60% ammonium sulphate precipitated, 100% acetone precipitated and 40% ethanol precipitated samples after dialysis and gel filtered sample using 4% w/v stacking gel and 12% w/v separating gel according to the procedure (Laemmli 1970) with Protein standard markers (14.4–116.0 kDa).

Native Polyacrylamide Gel Electrophoresis (Native-PAGE)

Native PAGE is regularly performed for analysis of homogeneity of the enzymes, which is different from SDS-PAGE. Native PAGE separates proteins according to their mass-charge ratio while in SDS-PAGE; SDS denatures and binds to the protein which is separated primarily by mass. Native PAGE is not a good measurement of molecular weight as SDS-PAGE but can determine the protein charge or subunit composition. Therefore, the analyzed enzymes (maintaining their natural conformation and homogeneity) can be determined by the method of (Sambrook and Russell, 2001).

Confirmation of Protease by Zymography

Casein zymography was done using a 12% SDS polyacrylamide gel containing 2% casein. Electrophoresis was performed at room temperature under non-reducing conditions. Following electrophoresis the gel was washed twice for 1 hour each in 100 ml of Triton X-100 to remove SDS and incubated for 24h at 31°C in 50mM Tris-HCl, 5 mM $CaCl_2$, 150mM NaCl, 0.05% Brij 35, pH 7.6 to allow proteolysis. The gel was stained with Coomassie Brilliant Blue R-250 and then destained.

RESULTS AND DISCUSSION

Purification profile of precipitated protease

Table (1) summarizes the protease activity, protein content, specific activity, purification fold and recovery % of crude enzyme, 40-60% ammonium sulphate precipitated, 100% acetone precipitated and 40% ethanol precipitated on comparison of the three precipitations. The protein content of the crude enzyme recorded the highest value (1.75 mg/ml) on comparison with all the other samples. The finding agrees with results of Liu *et al*., (2007) reported that the crude protease extract isolated from grass carp fish intestine shown the highest protein content when compared to 40-60% ammonium sulphate precipitated sample. Among the precipitation 40% ethanol showed the highest protein content. The protease activity (1.98 U/ml), specific activity (0.32 U/mg), purification fold (4.40) of 100% acetone showed the highest value than the other two samples. These data's agrees with (Subash *et al*., 2011), (Jellouli *et al*., 2009).

Table (2) records that the purification profile of protease precipitated samples 40-60% ammonium sulphate, 100% acetone, 40% ethanol gave a higher protease activity and specific activity is than further purified by dialysis and Sephadex G-100 gel filtration.

Purification by Dialysis

The protein content of the 40-60 % ammonium sulphate precipitated (0.75 mg/ml to 0.40 mg/ml), 100% acetone

Table 1. Purification profile of protease from visceral organ waste of *Lutjanus sebea* (Redfish)

Samples	Protein content (mg/ml)	Protease Activity (U/ml)	Specific Activity (U/mg)	Purification fold	Recovery %
Crude extract	1.75 ± 0.05	1.00 ± 0.06	0.09 ± 0.01	1.00 ± 0.00	100
40-60% $(NH_4)_2SO_4$	0.75 ± 0.05	0.94 ± 0.25	0.19 ± 0.00	1.95 ± 0.02	84.89
100% Acetone	1.40 ± 0.01	1.98 ± 0.13	0.32 ± 0.02	4.40 ± 0.04	80
40% Ethanol	1.60 ± 0.08	0.28 ± 0.08	0.29 ± 0.04	1.04 ± 0.00	88

Table 2. Purification profile of dialysates and Sephadex G-100 column purified 40-60% ammonium sulphate, 100% acetone and 40% ethanol precipitated protease from visceral organ waste of Redfish (*Lutjanus sebea*)

Samples		Protein content (mg/ml)	Protease Activity (U/ml)	Specific activity (U/mg)	Purification fold	Recovery %
Crude extract		1.75 ± 0.05	1.00 ± 0.06	0.09 ± 0.01	1.00 ± 0.00	100
40-60% ammonium sulphate precipitated	Dialyzed	0.40 ± 0.20	0.54 ± 0.40	1.06 ± 0.00	1.00 ± 0.12	24
	Sephadex G-100 column purified	0.3 ± 0.01	0.32 ± 0.02	1.24 ± 0.04	1.32 ± 0.03	16
100% acetone precipitated	Dialyzed	0.90 ± 0.30	1.24 ± 0.02	1.94 ± 0.008	2.30 ± 0.05	56
	Sephadex G-100 column purified	0.5 ± 0.05	1.04 ± 0.04	2.06 ± 0.03	2.60 ± 0.05	48
40% ethanol precipitated	Dialyzed	1.00 ± 0.20	0.20 ± 0.03	1.70 ± 0.00	1.30 ± 0.03	33
	Sephadex G-100 column purified	0.9 ± 0.02	0.10 ± 0.01	1.80 ± 0.03	1.60 ± 0.04	25

precipitated (1.20 mg/ml to 0.90 mg/ml) and 40% ethanol precipitated (1.60 mg/ml to 1.00) samples decreased on dialysis. When compared the protease activity, specific activity, purification fold and recovery % of the dialysed sample with the three precipitation methods, the protease activity and recovery % were decreased, specific activity and purification fold were increased. These findings are in accordance with the report of (Balti *et al.,* 2009), (Klomklao *et al*., 2011).

Purification by Gel filtration

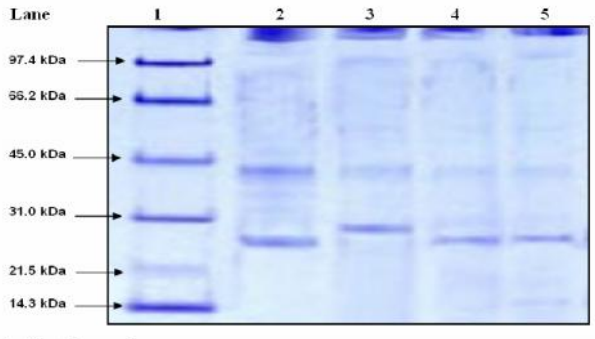
The dialysed sample was further purified by Sephadex G-100 column chromatography. Specific activity and purification fold of gel filtrated samples shows higher value, where the protease activity, protein content and recovery % has been decreased. These reports were in accordance to El-Beltagy *et al*. (2004).

Molecular Weight of Isolated Protease

The crude and partially purified samples of 40-60% ammonium sulphate precipitated, 100% acetone precipitated and 40% ethanol precipitated samples were analyzed by SDS-PAGE to determine the molecular weight of the isolated enzyme. The molecular weight of the isolated protease enzyme was found to be 27KDa when compared to the standard marker protein of 14.3-97.4 KDa. The thickness of the band indicates that there is an increase in the purity level in the Sephadex G-100 column eluted sample. These findings are supported by the study of (Liu *et al*., 2007) who reported that the molecular weight of protease from fish waste of grass carp intestine was around 27 kDa. This study also agrees with the report of (Yanez et *al.*, 2005) who stated that purified proteolytic enzyme chymotrypsin from the viscera of Monterey Sardine fish showed a molecular weight of 27 kDa.

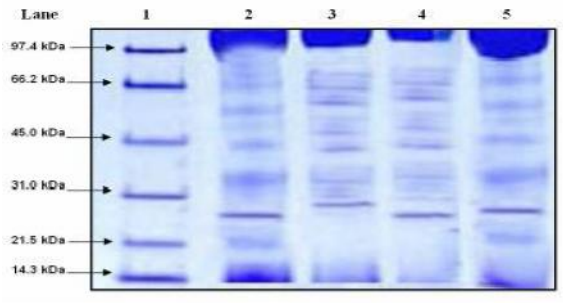
Enzyme homogeneity of isolated protease

The Sephadex G-100 column purified 40-60% ammonium sulphate precipitated, 100% acetone precipitated and 40% ethanol precipitated sample was analyzed by Native-PAGE to determine the homogeneity of the enzyme.



Lane 1- Protein marker
Lane 2- Crude
Lane 3- 40-60% Ammonium sulphate purified sample
Lane 4- 100% Acetone purified sample
Lane 5- 40% Ethanol purified sample

Figure 1. SDS-PAGE gel pattern of crude and partially purified 40-60% ammonium sulphate precipitated, 100% acetone precipitated and 40% ethanol precipitated samples



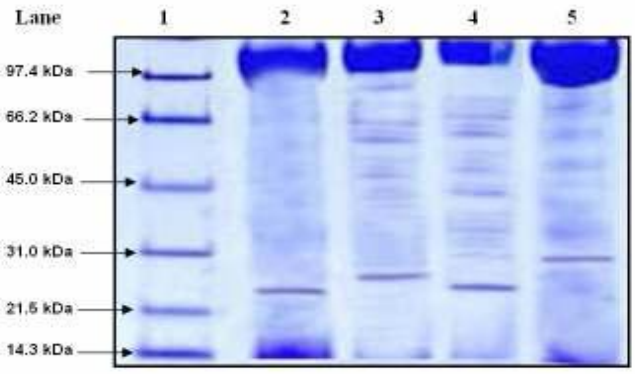
Lane 1- Protein marker
Lane 2- Crude
Lane 3- 40-60% Ammonium sulphate purified sample
Lane 4- 100% Acetone purified sample
Lane 5- 40% Ethanol purified sample

Figure 2. NATIVE-PAGE gel pattern of purified 40-60% ammonium sulphate precipitated, 100% acetone precipitated and 40% ethanol precipitated samples

Figure (2) shows the banding patterns of the Sephadex G-100 column purified samples. From the figure a single band corresponding to around 27 kDa to the protein marker can be seen in lane 2 and 3. Thus it can be deduced from the banding pattern that the molecular weight of the enzyme on Native-PAGE is around 27 kDa. This study agrees with the findings of Bhaskar *et al*. (2007) who stated that purified acidic protease from Bolti fish showed a molecular weight of 27 kDa.

Confirmation of presence of protease

The proteolytic activity of the enzyme was confirmed on an activity gel/zymogram.



Lane 1- Protein marker
Lane 2- Crude
Lane 3- 40-60% Ammonium sulphate purified sample
Lane 4- 100% Acetone purified sample
Lane 5- 40% Ethanol purified sample

Figure 3. Zymography gel pattern of partially purified 40-60% ammonium sulphate, 100% acetone and 40% ethanol precipitated samples

The zymogram showed clear bands indicating the caseinolytic activity of the protease enzyme isolated from the fish waste. The enzyme isolated from the fish *Lutjanus sebea* is active only in its monomeric form, having a molecular weight of 27 kDa as determined on native page. On conclusion, the aim of this research work was to isolate and purify protease from the visceral organ waste of *Lutjanus sebea* precipitated with ammonium sulphate, acetone and ethanol, purify them by dialysis and Sephadex G-100 column chromatography. The partially purified enzyme had molecular weight about 27 kDa. Zymography was done for confirmation of presence of protease. From the above observations, 100% of acetone is the best method for purification technique when compared to other solvent and salting-out method. Further studies are in progress in the characterization of the isolated protease and its application in different commercial fields.

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