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RESEARCH ARTICLE

PURIFICATION AND CHARACTERIZATION OF PROTEASE ENZYME FROM SEA WEED, GRACILARIA FERGUSONII

[*1]Rama Devi, P., [2]Babu, C., [1]Vasudhevan, I., [1]Felcial, S. and [3]Lakshmanan, G

[1]Aditanar College of Arts and Science, Tiruchendur - 628 216, Tamil Nadu, India
[2]Pioneer Kumaraswamy College, Nagercoil - 629003, Tamil Nadu, India
[3]Vivekananda College, Agasteeswaram, Kanyakumari-629701, Tamil Nadu, India

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ABSTRACT

In the present study protease enzyme was purified from sea weed, *Gracilaria fergusonii* by gel filtration chromatography using Sephadex G-75 and their molecular mass was determined on SDS-PAGE. The protease enzyme activity was initially verified by protease assay. The enzyme was highly active in 2.0 % of substrate concentration and $CaCl_2$ necessary to their enzyme activity. The optimum temperature at 50ºC and pH at 8.

Key words: Sea weed, Enzyme, Purification, Chromatography, SDS-PAGE.

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INTRODUCTION

Proteases are hydrolytic enzymes which catalyse the cleavage of specific peptide bonds in their target proteins. They are essential for various cellular and metabolic processes, such as sporulation and differentiation, cell migration and invasion, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Proteases execute a large variety of functions from the cellular level to the organ and organism level, to produce cascade systems like homeostasis and inflammation; and complex processes involved in the normal physiology as well as in abnormal patho physiological conditions. They have also gained considerable attention in the industrial community mainly in detergent, pharmaceutical, food, diagnostics, leather, waste management and silver recovery sectors (Gupta *et al*., 2002). Proteases being ubiquitous are found in a wide diversity of sources such as plants, animals, and microorganisms (Maghsoodin *et al.,* 2013) and seaweeds (Patil and Rebecca, 2014). Microbial proteases account for approximately 40% of the total world wide enzyme sales (Godfrey and West, 1996). A large number of enzymes and enzyme systems have been detected from plant tissues and extracts; subsequently, many of these have been isolated and purified. In addition certain fresh water algae have been widely used in studies of photosynthesis and intermediary carbohydrate metabolism. By contrast, similar information on marine algae is lacking and few investigations of the enzyme systems of marine algae have been reported, although the chemical structure of many of the end products of anabolism has been investigated (Black,1953). But very limited number of work done on enzyme production of sea weeds. So the present research work aim to study purification and characterization of protease enzyme from sea weed, *Gracilaria fergusonii.*

Corresponding author: **Rama Devi,**
Aditanar College of Arts and Science, Tiruchendur - 628 216, Tamil Nadu, India

MATERIALS AND METHODS

Collection of sample: The greenish red sea weed, *Gracilaria fergusonii* was freshly collected in sterile bottle from Manapadu, Thoothukudi District. The collected sea weeds were washed with distilled water and stored at -20ºC for further study.

Fig. 1. The sea weed, *Gracilaria fergusonii*

Preparation of sample: 5 g of sea weed was cut into small pieces and were ground well with 25 ml of Tris buffer (pH 7.6) with the help of mortar and pestle. The crude mixture was filtered using Whatman No.1 filter paper and stored at -4°C for further use.

Protease assay: Protease assay was carried out as by following the method Rama Devi *et a1*. (2004). 2 ml of the sample was mixed with 5 ml of substrate (1.0% casein) and incubated for 1 hr at 37°C. To the reaction mixture 5.0 ml of TCA was added and incubated for 30 mins at 37°C. Mixed well and filtered. 2.0 ml of filtrate was taken and 5.0 ml of sodium carbonate solution, 1.0ml of folin phenol reagent was added. Then it was incubated at 37°C for 30 min and observed the optical density at 660nm.

Protein quantification: Protein concentration was determined following by the method of Lowry *et al.* (1951) using the protein assay kit and bovine serum albumin as the standard. 1.0 ml of reagent taken in 20µl of sample. It was incubated for 10 min at 37°C and the absorbance was measured at 560 nm. The standard curve was documented and the protein concentration of the samples was calculated from the standard curve.

Characterization of proteases enzyme activity

Effect of temperature: The sample was incubated at various temperatures such as 30, 40, 50, and 70°C and then the effect of temperature on enzyme production was observed. The optimum temperature of enzyme activity was noted.

Effect of pH: Different pH of buffers (Sodium acetate buffer with pH 4.5 – 5.8, and phosphate saline buffer with pH 6.0 – pH 7.0 and Tris buffer saline buffer with pH 7-12) were prepared. The protease assay used this buffers. The optimum pH of enzyme activity was noted.

Effect of substrate concentration: The different concentrations of casein (0.5, 1.0, 1.5, 2.0 and 2.5%) was prepared and used the enzyme activity. The optimum concentration of maximum activity was noted.

Effect of cations: The effect of cations on enzyme activity was studied by incubating 1mM of cations with the sample. $CaCl_2$, $MnSO_4$, $HgCl_2$, and $CuSO_4$. were used the enzyme activity.

Ammonium sulphate precipitation: The ammonium sulphate precipitation method was carried out by following the method of Murali *et al.* (1994). 10 ml of sample was mixed with 10% ammonium sulphate and was kept in 4^0C for 4 hrs. Then it was centrifuged at 4,000 rpm for 10 mins. The supernatant was transferred into 20% ammonium sulphate and was continued upto 90%. The pellets were stored at -20^0C.

Dialysis: Cut dialysis membrane to the required length and soaked in distilled water for 15 min. Then the membrane was transfer into 10 mM sodium bicarbonate and boil for 10 min at 80^oC. Next the membrane wash several times in distilled water and transfer the membranes into a 10 mM Na_2EDTA solution and boil for 10 min at 80^oC. Again boiled in distilled water and stirred for 30 min at 80°C. Tide one end of the membrane tightly use tread, add the sample into the bag use micropipette and tide the other end tightly. The dialysis bag overnight in Tris buffer under the refrigerator at 4^oC and frequently exchange the buffer. The dialysis sample centrifuge for 15 min at 12,000 rpm in a 4°C. Supernatant transfer into another tube and store at -20^oC.

Purification of protease enzyme
Gel filtration chromatography

Preparation of gel filtration matrix Sephadex G-75: The gel filtration chromatography was carried out by following the method of Sudhakar *et al*. (2012). 2 g of Sephadex G-75 powder was stirred well with distilled water and allowed to settled. The fine particles were removed from the supernatant by suction. The slurry was equilibrated with 50 mM TBS buffer (pH 7.6) and stirred well and allowed to settled and the supernatant was decanted. The elution buffer (TBS with 50 mM Tris, 140 mM NaCl, pH 7.6) was added to the gel at 4 or 5 times to settle the gel volume. The Sephadex G-75 gel was gently stirred and allowed to settle and fine particles were removed. Addition of buffer gel settled and decantation were repeated three times. The equilibrated gel was kept at 4^oC overnight before packing the columns.

Gel filteration column packing and elution: The swelled gel was degassed and packed in the glass column (0.6 × 100 cm). Sephadex G-75 gel was uniformly packed without air bubbles. Then the gel was equilibrated with elution buffer. After equilibration, the dialyzed sample was applied to the column. Elution was done with the help of elution buffer and the 25^{th} fractions were collected at the flow rate of 2 ml / 20 mins in propylene tubes. The OD value of each fraction was observed at 280 nm. Enzyme active fractions were identified through the enzyme assay and protein content was estimated using Lowry *et al*. (1951) method.

Molecular weight determination

SDS-PAGE was carried out by the method of (Gam and Latiff, 2005). Protease was denatured with the sample buffer containing 1% SDS by boiled for 3min at 100^0C. Treated sample was loaded into 12% SDS-PAGE on the electrophoresis set. Electrophoresis was carried out at 50vots initial current. When the stacking dye reaches the separating gel, the current was increased to 100vots. The molecular mass of the proteas was estimated by comparing its mobility with that of the following markers. Phosphorylase b (97400), Bovine Serum Albumin (66,000), Ovalbumin (43,000), Carbonic Anhydrase (29,000), Lactoglobulin (18,400), Aprotinin (6,500), Gel was silver stained by the method of (Blum et *al.,* 1987).

RESULT AND DISCUSSION

A number of enzymes and enzyme systems have been detected from sea weed extracts; subsequently, many of these have been isolated and purified. In this present study the protease enzyme was purified and characterized from sea weed, *Gracilaria fergusonii*. The protease enzyme activity was initially verified by protease assay. Then study the effect of substrate concentration on protease activity was investigated. The tested concentrations (0.5, 1.0, 1.5, 2.0, and 2.5)**.** The results showed (Fig.2) that enzyme activity high in 2.0 % of substrate concentration. The optimum pH range of alkaline proteases is generally between 9 and 11, with a few exceptions of higher pH optima of 11.5, 11–12, 12.3 and 12–13 (Rao *et al.,* 1998). In the present study the protease activity was assayed with various pHs. This enzyme was stable at the pH ranges between 7.0 and 8.0. The enzyme activity reduced greatly from pH 8.0. Enzyme activity was observed at lower pHs also but it was less. No activity was observed at pH below 4.5. These results are in harmony with those reported for the enzyme from *Ulva fasciata* (Patil and Rebecca, 2014) and *Ulva pertusa* (Kang *et al*., 2015). Jellouli *et al*. (2009) similarly reported the optimum pH to be 7.0 for protease activity. The importance of neutral proteases are their application in the food industry, because

they perform specific function reducing the bitterness of food protein hydrolysates through hydrolyzing hydrophobic amino acid bonds at neutral pH (Sandhya *et al.,* 2005).

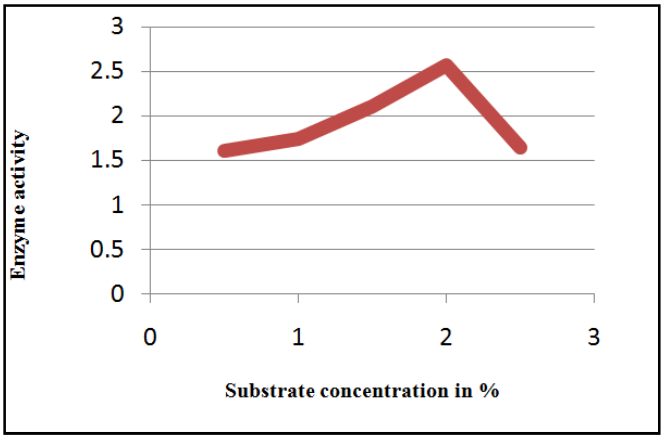

Fig. 2. Effect of substrate concentration on enzyme activity of sea weed, *G. fergusonii*.

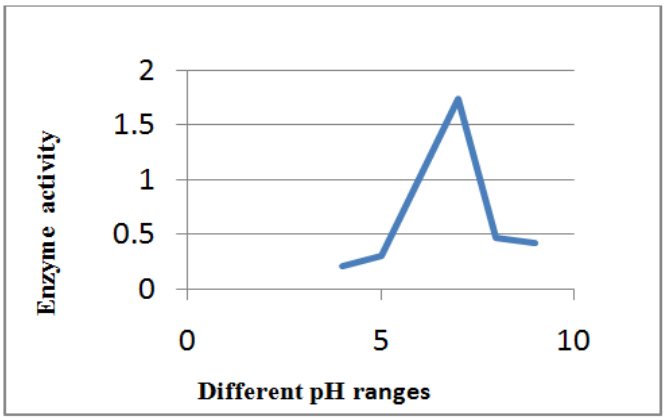

Fig. 3. Effect of pH on enzyme activity of sea weed, *G. fergusonii*

The enzyme is highly stable over a range of temperature, however it is found to be most stable at around 20^0 C (Shiladitya Mitra *et al*., 2011). In this present study optimum temperature (20, 30,40, 50, 60, and 70 ºC) on protease activity was investigated. The results showed in Fig.4 indicate that there was continuous increase in the enzyme activity with increasing the temperature up to 50ºC after which the activity declined at 50, 60 and 70ºC. Therefore, the optimal temperature was 50ºC. The results are consistent with those of for the enzyme from *U. fasciata* (Patil and Rebecca, 2014) *U. pertusa* (Kang *et al.,* 2015). Alkaline proteases requires a divalent cation like $Ca^{2+,}$ Mg^{2+} and Mn^{2+} or a combination of these cations, for maximum activity. In the present study sea weed extract was incubated with various cations ($MgCl_2$, $MnCl_2$, $CaCl_2$ and EDTA) in 1Mm concentration and the protease activity was assayed.

The results (Fig.5) indicate that cation $CaCl_2$ and $CuSo_4$ activated the protease activity. The $CuSo_4$ was better activator than $CaCl_2$. It is believed that these cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures as they impart structural and thermal stability to the protein (Pan and Lin, 1991). In addition, specific calcium binding sites that influence the protein activity and stability apart from the catalytic site were described for Proteinase K (Bajorath *et al.,* 1988).

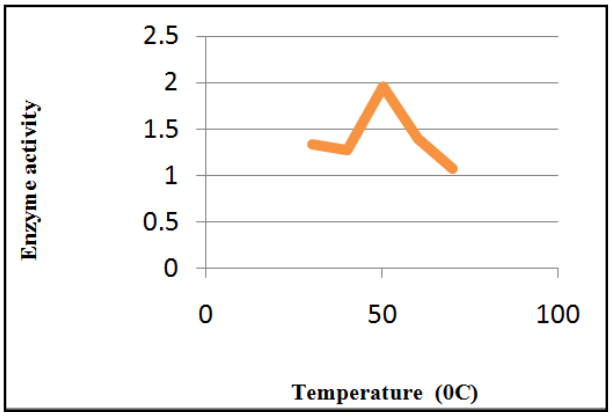

Fig. 4. Effect of temperature on enzyme activity of sea weed, *G. fergusonii*

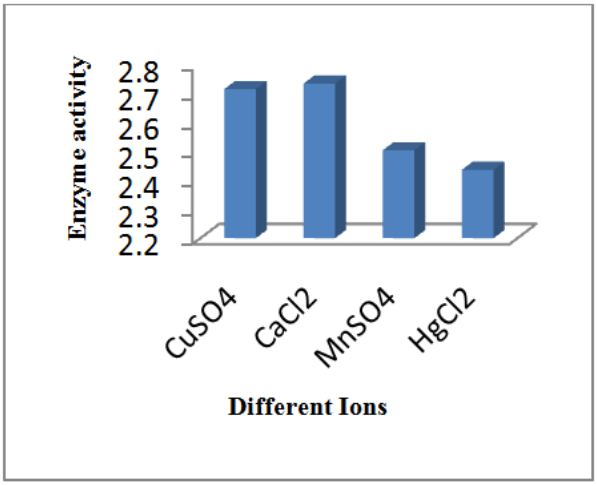

Fig. 5. Effect of divalent cations on enzyme activity of sea weed, *G. fergusonii*

Ammonium sulphate is the commonly used salt for protein precipitation, though sodium sulphate is also rarely used. Sodium sulphate has better precipitating qualities over ammonium sulphate, but due to poor solubility at low temperatures its use is restricted (Shih *et al.,* 1992). In the present study protease was partially purified by ammonium sulphate fractionation (10%, 20%, 30%, 40%, 50% and 90% of saturation). Among this 70 % ammoniam sulphate precipitate only given the protease activity.

Table 1. Protein estimation in sea weed, *G. fergusonii* crude sample, ammonium sulphate precipitate and purified sample

Sample	Protein (µg/ml)
Sea weed crud sample	8.606
Ammonium sulphate precipitation	4.969
Purified sample	7.03

Purification of proteases to homogeneity is a prerequisite to study their mechanism of action and behavior. There are various methods adopted for the purification of proteases like ultra filtration, chromatography, preparative gel electrophoresis and so on. Various chromatographic techniques like ion exchange chromatography (IEC), affinity chromatography (AC), hydrophobic interaction chromatography (HIC) and gel filtration chromatography are most widely used (Kumar and Takagi, 1999). The present investigation showed that the protease enzyme purified by gel filtration column chromatography using Sephadex G-75. The

similar study was conducted by Shora *et al*. (2016) purified the protease enzyme from *Petrocladia capillacea* by gel filtration chromatography on Sephadex G-200. The mass density of proteins is an important basic biophysical quantity, which is directly related to the volume of a macromolecule of a given molecular weight. It is also a useful input parameter for macromolecular structure determination in X-ray single crystal crystallography. The molecular weight can be determined by SDS-PAGE used to the molecular weight of an unknown protein sample. The present study sea weed, *G. fergusonii* molecular weight determined by SDS-PAGE analysis. The enzyme molecular weight is 39 kDa.

Conclusion

Protease enzyme was successfully purified from the seaweed of *Gracilaria fergusonii.* The sea weed extract the showed positive result to confirming the presence of protease. The optimum temperature and pH of protease were 7.0 - 8.0 and 50^{o}C, respectively. This present study, it is quite evidence that the sea weed, *G. fergusonii* have valuable source of protease. So this research work highly recommend that the sea weed, *G. fergusonii* could be use in food industrial purpose.

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