

Original Research Article

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Purification and Biochemical Characteristic of Protease from the Red Seaweed *Petrocladia capillacea*

Hamed M El-Shora*, G.S. Abou-El-Wafa and A.I. Kadhim

Botany Department, Faculty of Science, Mansoura University, Egypt

*Corresponding author

ABSTRACT

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The aims of the present work was to purify protease enzyme from the red seaweed *Petrocladia capillacea*. Protease was purified by 70 % ammonium sulfate and Sephadex G-200. The specific activity was 280 units mg⁻¹ protein and with 122-fold. The optimal casein concentration was 40% (w/v). The K_m and V_{max} values were 52.6 % (w/v) and 33.3 units mg⁻¹ protein. The final incubation time was 40 min. The optimal pH and optimal temperature were 7 and 40°C. Ca⁺² and Mg⁺² were the best activator divalent cations. The other cations Hg²⁺, Cu²⁺, Al³⁺, Co²⁺ and Zn²⁺ were inhibitors. Protease was activated by the amino acids cysteine and methionine. However, protease was activated by reduced glutathione, thiourea, thioglycolate, and acetyl cysteine.

Introduction

Seaweeds are multicellular macroalgae used as a potential renewable resource in the field of medical and commercial environment. Many seaweeds have commercial applications in pharmaceutical, medical, cosmetic, nutraceutical, food and agricultural industries (Torres *et al.*, 2014).

Seaweeds are very common for people since they are rich in vitamins and minerals mainly calcium, sodium, magnesium, potassium, iodine, iron, and zinc (Lopez *et al.*, 2009; Caliceti *et al.*, 2001).

Enzymes are biocatalysts that have been used as alternatives to chemicals to improve the efficiency and cost-effectiveness of a wide range of industrial processes.

They are currently used in applied and basic areas of research as well as in a wide range of products and manufacturing processes, such as food, beverage, detergent, pharmaceutical, leather processing and peptide synthesis industries (Gupta *et al.*, 2002).

Proteases have a wide distribution in plants (Sharmila *et al.*, 2012), microorganisms (Maghsoodi *et al.*, 2013) and seaweed (Patil and Rebecca, 2014).

Proteases constitute about 70% of industrial enzymes and microbial sources are leading suppliers of these enzymes. These enzymes possess catalytic activity in broad

range of temperature and pH (Murakami *et al.*, 1991; Sanatan *et al.*, 2013).

The protease is also called as peptidase or proteinase is group of enzyme that performs hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein(Hedstrom, 2002).

The present work aimed to purify protease from the red seaweed *Petrocladia capillacea*. It is also aimed to study the biochemical characteristics of the enzyme.

Materials and Methods

Collection of Seaweed

Petrocladia capillacea samples were collected by hand from submerged rocks on the coast of Abu Qir Bay, Alexandria, Egypt. After collection, the samples were transported to the laboratory in plastic bags containing sea water to prevent evaporation

Cleaning of Algae

The algal samples then cleaned from epiphytes and rock debris then rinsed many times in distilled water to remove surface salts. Some of the collected samples were preserved for identification. The seaweed was identified following Aleem (1993).

Preparation of Algal Protease Extract

A fresh sample (0.5 g) of algae was suspended in 100 ml phosphate buffer (pH 7.0) the buffer containing 1 mM dithiothreitol, 10 mM glycine and processed for 10 min in pestle and mortar. The resulting homogenates were then pooled and filtered through cheesecloth the filtrate is used as crude extract.

Assay of Algal Protease

The purified enzyme (0.5 ml) was mixed

with 0.5 ml casein. The reaction mixture was incubated for 1h at 40°C and stopped by addition of 1 ml of 15% TCA. The mixture was then centrifuged and supernatant was collected. The supernatant was mixed with Na₂CO₃ and NaOH and Folin Phenol. This mixture was shaken and the absorbance was taken at 650nm. One protease unit was defined as the amount of enzyme that releases 0.5 µg/ml/min tyrosine. The protease activity was measured spectrophotometrically.

Determination of Protein Content

Soluble protein content was determined as described by Bradford (1976). Ten g of *Petrocladia capillacea* were homogenized in 100 mM phosphate buffer pH (7.0). The extract was centrifuged for 30 min at 6,000 rpm. One ml of supernatant was added to 5ml diluted Coomassie Brilliant Blue G-250 and vigorously mixed. After keeping it in the dark for 1 min, the absorption of the protein in the extract at 595 nm was spectrophotometrically measured. The protein concentration was determined from standard curve using bovine serum albumin.

Purification of Algal Protease

Ammonium Sulphate Fractionation

The crude algal was homogenated with ammonium sulphate. The supernatant was saturated up to 70% with (NH₂)₂SO₄ under continuous at pH 7. The suspension was centrifuged at 8,000 g for 15 min at 0-4°C. The pellet obtained was dissolved in extraction buffer (20 mM, pH 7.0). The final volume was made up to 10 ml. This fraction was extensively dialyzed using dialysis tube with continuous stirring for 24 hours at 0-4°C against the same buffer.

Gel filtration chromatography

The dialyzed extract was further purified

using filtration chromatography (Sephadex G-200). The Sephadex beads were activated using extraction buffer for 72 hours with washing several times. Activated beads were poured slowly in glass chromatography column with the help of a glass rod to avoid trapping of air bubbles. Eluents were collected in different fraction tubes of 1 ml. The fractions containing higher activity were pooled were diluted with extraction buffer according to need. The obtained enzyme was stored at 0-4 °C and used throughout this study.

Estimation of Kinetic Parameters

The Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) were determined by investigating the effect of casein concentration on enzyme activity. Enzyme activity was determined at different casein concentrations (10-50 % w/v). The Lineweaver-Burk plot ($1/V$ against $1/S$, where V is the reaction velocity) was then constructed. The K_m and V_{max} were determined for algal protease from this graph.

Effect of pH on Algal Protease

The optimal pH for enzyme activity was investigated over a range from 4-10. Sodium acetate/acetic (pH 3.0 – 4.0 - 5.0), potassium phosphate buffer (6.0 – 7.0), Tris- HCl buffer (8.0 – 9.0) and sodium bicarbonate buffer (10.0) were used. From the results obtained a graphic of enzyme activity vs pH was plotted, and the optimum pH for algal protease was determined.

Effect of Temperature on Algal Protease

Reaction mixture was incubated at different temperatures 20, 30,40,50,60, 30 °C. The optimum temperature was determined from the graphic of enzyme activity against. The enzyme activity was estimated by the

method described above.

Effect of Metal Cations on Algal Protease

The effect of divalent cations Mg^{+2} , Ca^{+2} , Co^{+2} , Zn^{+2} , Al^{+2} , Cu^{+2} and Hg^{+2} (in the form of their chlorides) at 5 mM on the activity of the algal protease was studied by including them in the standard reaction mixture containing the enzyme and casein as substrate. The control was done with enzyme without metals.

Effect of Amino acids on Algal Protease

The effect of the amino acids cysteine, methionine, lysine, glutamic acid, arginine, tyrosine and glutamine on protease activity was carried out in the reaction mixture at 5 mM.

Effect of Thiol Compounds on Algal Protease

The effect of the thiol compounds reduced glutathione (GSH), thiourea, thioglycolate, and acetyl cysteine on algal protease was investigated in the reaction mixture. GSH was tested at 10-50 mM, however thiourea, thioglycolate and acetyl cysteine were tested at 20-100 mM.

Results and Discussion

Purification of Algal Protease

Protease was purified from *Petrocladia capillacea* by ammonium sulfate and Sephadex G-200 with specific activity of 280 units mg^{-1} protein with 122-fold (Table 1). Protease from *Bacillus amyloliquefaciens* H11 was purified with 16-fold (Sai-Ut *et al.*, 2015).

Effect of Casein on Protease Activity

The effect of casein concentration (% w/v) on protease activity was investigated. The tested concentrations (10, 20, 30, 40, and 50

(% w/v). The results in Fig. 1 indicate that by increasing the casein concentration the activity was continuous increase in the activity up to 40 % (w/v) after which there was steady increase at 50 % (w/v). K_m was 52.6 % w/v and V_{max} was 33.3 units mg^{-1} protein (Fig. 2).

Effect of pH on Protease Activity

The results in Fig. 3 show the optimal pH value was 7.0. 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.* (2011) 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). However, optimal pH 9.0 was reported for the enzyme from *Bacillus subtilis* (Naidu, 2011).

Effect of Temperature on Protease Activity

In this experiment of temperature (20, 30, 40, 50, 60, and 70 °C) on protease activity was investigated. The results in Fig.4 indicate that there was continuous increase in the enzyme activity with increasing the temperature up to 40°C after which the activity declined at 50, 60 and 70°C. Therefore, the optimal temperature was 40°C. The results are consistent with those of for the enzyme from *Ulva fasciata* (Patil and Rebecca, 2014) *Ulva pertusa* (Kang *et al.*, 2015). However, optimum temperature of 60 °C was reported for the enzyme from *Bacillus subtilis* (Naidu, 2011).

Effect of Incubation Time on Protease Activity

In this experiment the relation between

incubation time and protease activity was investigated. The protease activity was assayed throughout various time intervals (10, 20, 30, 40, 50, and 60 min). The results in Fig.5 show that there was a corresponding increase in the enzyme activity with increasing the time. The optimum time was 40 min after which the activity declined gradually.

Effect of Metal Ions on Protease Activity

The effect of metals ions Ca^{+2} , Al^{+3} , Mg^{+2} , and Zn^{+2} on protease activity. These cations were tested at 5 mM. The results in Fig.6 indicate that cation Ca^{+2} and Mg^{+3} activated the protease activity. The Ca^{+2} was better activator than Mg^{+2} . This in accordance with the results of Ahmetoglu *et al.* (2015) for the enzyme from *Bacillus sp.* KG5.

Adinarayana *et al.* (2003), Hmidet *et al.* (2009) and Annamalai *et al.* (2014) stated that Ca^{2+} acts as an activator for proteases. The increase in the activity in the presence of Ca^{2+} may be attributed to stabilisation of enzymes in its active conformation rather than it being involved in the catalytic reaction. It may possibly acts as a salt or an ion bridge via a cluster of carboxylic groups (Divakar *et al.* 2010). The other remaining metal ions inhibited the activity. The inhibition of protease by Cu^{+2} and Zn^{+2} is similar to the enzyme from *Ulva pertusa* (Kang *et al.*, 2015). The protease from *Brevibacillus (Bacillus brevis)* was also inhibited by Hg^{2+} , Zn^{2+} and Cu^{2+} (Banerjee *et al.*, 1999). These metal ions chemically react with the protein thiol-group in addition to tryptophan and histidine amino acid in the polypeptide chain of enzyme. Furthermore, the disulphide bonds were noted to hydrolytically breakdown in the presence of mercury and silver metal ions (Ladenstein and Antranikian, 1998).

Table.1 Purification Steps of Neutral Protease from *Petrocladia capillacea*

Purification steps	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Fold of purification	Yield (%)
Crude enzyme	644	280	2.3	1	100
70 % Ammonium sulfate	274	4.0	68.5	29.8	42.5
Sephadex-G200	140	0.5	280	122	21.7

Fig.1 Effect of Casein Concentration on Neutral Protease Activity

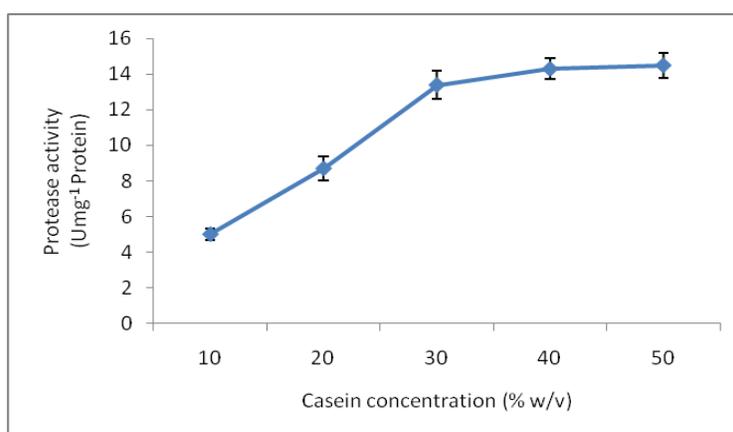


Fig.2 Lineweaver-Burk Double Reciprocalplot for Neutral Protease

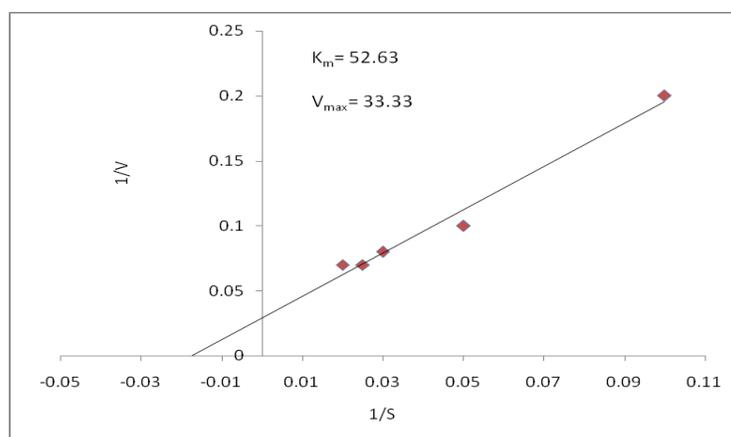


Fig.3 Effect of pH on Neutral Protease

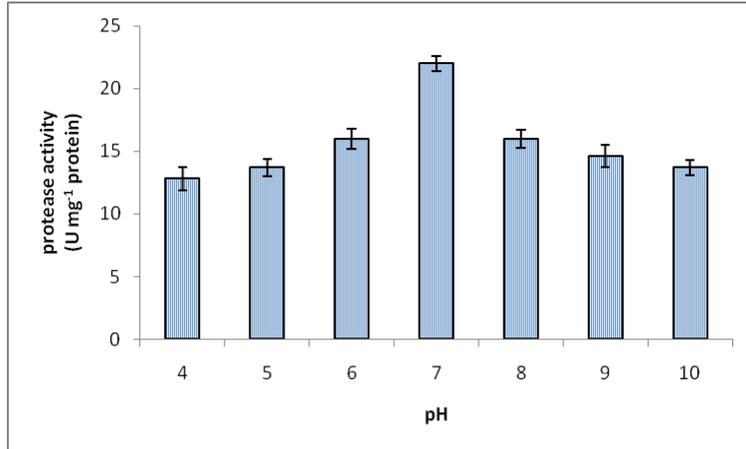


Fig.4 Effect of Temperature (°C) on Neutral Protease Activity

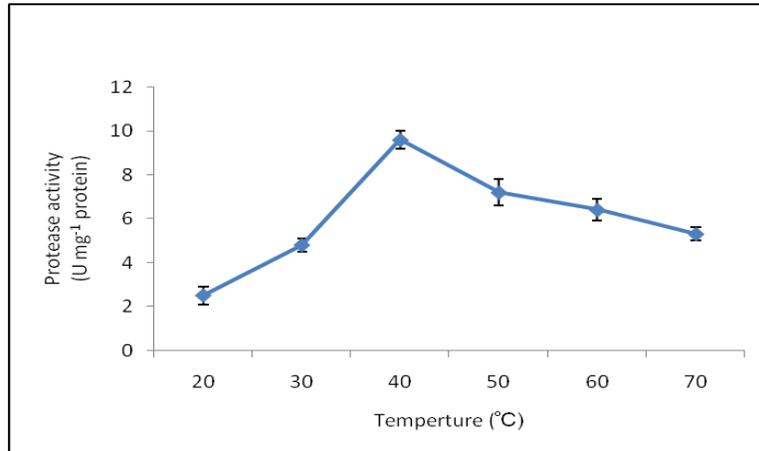


Fig.5 Effect of Incubation Time on Neutral Protease Activity

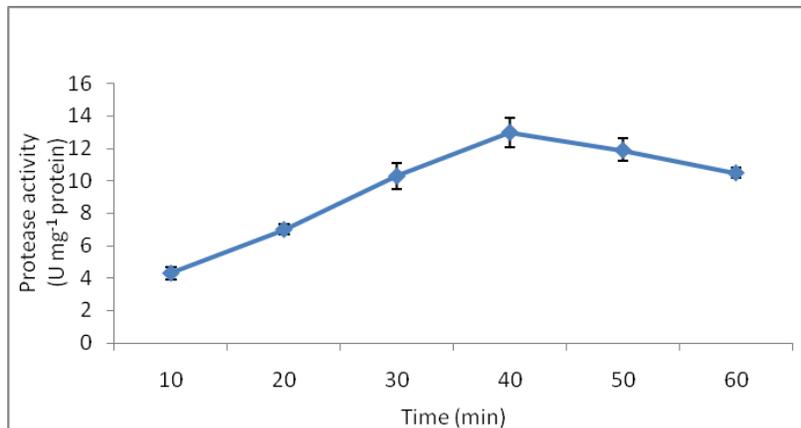


Fig.6 Effect of Metals Ions on Neutral Protease Activity

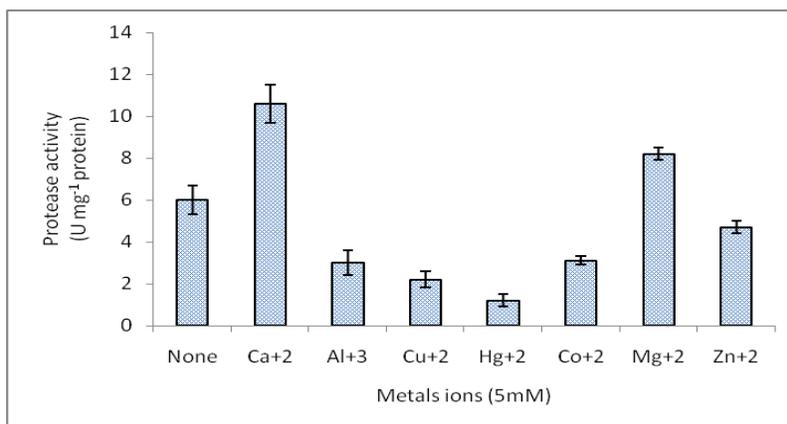


Fig.7 Effect of Some Amino Acids on Neutral Protease Activity

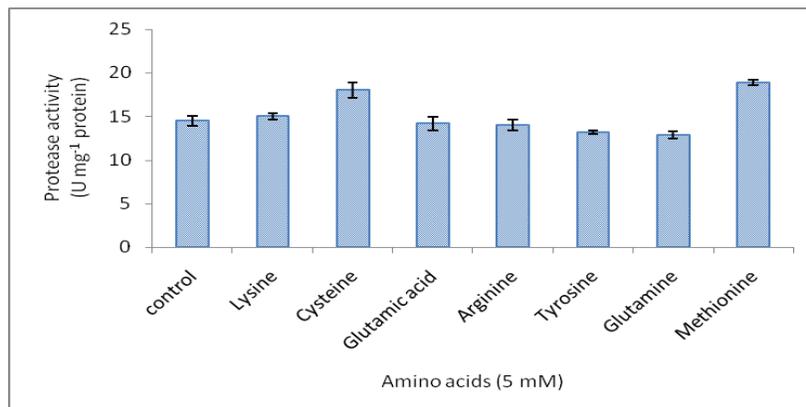


Fig.8 Effect of GSH on Neutral Protease Activity

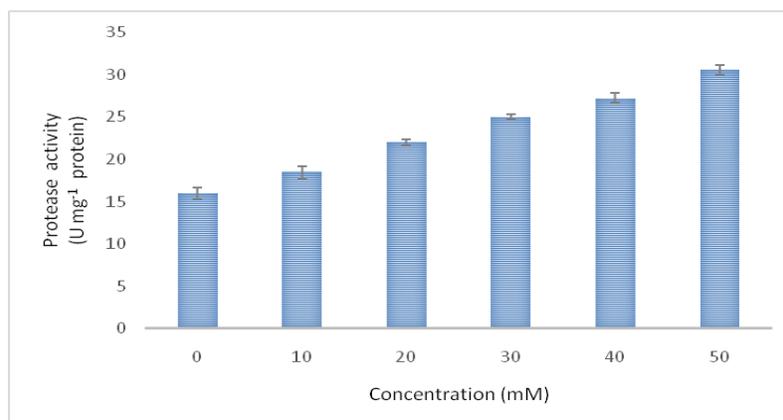


Fig.9 Effect of Thiourea on Neutral pPotease Activity

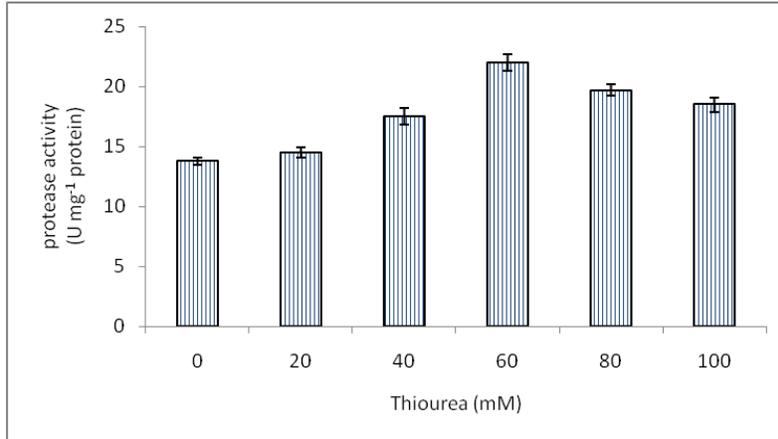


Fig.10 Effect of Thioglycolate on Neutralprotease Activity

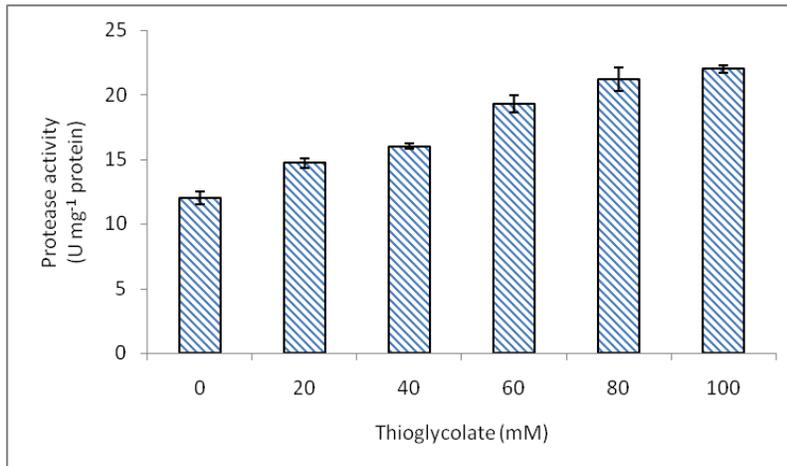
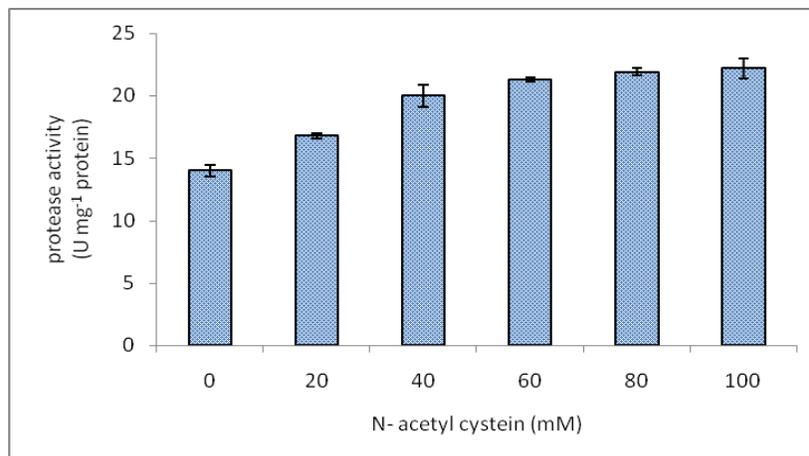


Fig.11 Effect of N- acetyl Cysteine on Neutralprotease Activity



Effect of Various Amino acids on Protease Activity

The effect of various amino acids on protease activity was investigated. The amino acids studied were lysine, glutamine and methionine. These amino acids were tested at 5mM. The results obtained are shown in Fig.7. These results indicate that cysteine and methionine as sulfhydryl containing amino acid activated the enzyme activity. The activity with these two amino acids were 18.0 and 18.9 units mg^{-1} protein. Cysteine activated other enzymes such as asparaginase (Warangkar and Khobragade, 2010). The activation by cysteine and methionine also may reveal the importance of these amino acids to protect the enzyme conformation and protect sulfhydryl group. Lysine did not show any remarkable effect on the enzyme. The other remaining amino acids expressed an inhibitory effect varies from one amino acid to the other. The inhibition of protease by the amino acids may be possibly due to competitive inhibition.

Effect of GSH on Protease Activity

In this experiment the effect of reduced glutathione (GSH) on protease activity was investigated. This compound was tested at various concentration (10, 20, 30, 40, and 50 mM). The results obtained are shown in Fig.8. These results show increasing the enzyme activity in concentration-dependent manner. The initial activity was 16 units mg^{-1} protein which increased gradually up to 30.6 units mg^{-1} protein at 50 mM. GSH activated other enzymes such as asparaginase (Warangkar and Khobragade, 2010). The molecular mechanism behind the activation effects of enzymes by glutathione have been the scope of the investigations for researchers. Enzyme activation by glutathione is not an uncommon event in enzymology. For example, catalytic activity

of protease from *Schistosoma mansoni* (Chappell, 1987), phosphoarginine hydrolase from rat liver (Kuba *et al.*, 2005) and reticulocytic protein kinase (Ernst *et al.*, 1979) were activated by the addition of GSH.

Effect of Thiourea on Protease Activity

In this experiment the effect of the thiourea on the protease activity at various concentration (20, 40, 60, 80 and 100 mM) was investigated. The results are shown in Fig.9. These results indicate that there was continuous increase in the enzyme activity to 60mM where the activity was 22 units mg^{-1} protein, after which the activity was the enzyme declined. Thiourea activated other enzymes such as asparaginase (Warangkar and Khobragade, 2010).

Effect of Thioglycolate on Protease Activity

This experiment was carried out to study the effect of various concentrations (20, 40, 60, 80, and 100). The results are shown in Fig.10. These results indicate that there was relationship between the thioglycolate concentration corresponding reaction and the enzyme activity. Thioglycolate can activate the enzyme by maintaining the thiol group in the reduced state (Beena *et al.*, 2010).

Effect of N- acetyl cysteine on Protease Activity

This experiment was designed to investigate the effect of N- acetyl cysteine on protease activity. This compound was tested at various concentrations (20, 40, 60, 80, and mM). The results in Fig.11 show that as the concentration of this compound increased the enzyme activity increased up to 80 units mg^{-1} protein after which there was steady increase at 100 mM. The enzyme was

activated by thiol-containing compounds including reduced glutathione, thiourea, thioglycolate and N- acetyl cysteine. The activation by thiol compounds is in harmony with the results of Kamran *et al.*, (2015) who found that mercaptoethanol as another thiol-compound stimulated protease from thermophilic *Bacillus* sp. This may indicate that SH- group of the enzyme was protected during the incubation time. Also, it is possible that these thiol compounds may lower the K_m of the enzyme to its substrate and thus activating the activity.

In conclusion, a protease was successfully purified from the *Petrocladia capillacea*. The optimum temperature and pH of protease were 7.0 and 40°C, respectively. Thus, protease could be applied in many industrial purposes. Biotechnological and commercial enzymes such as neutral proteases produced by *Petrocladia capillacea* are of importance in the food industry. This is because they perform specific functions reducing the bitterness of food protein hydrolysates.

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