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Original Article

Isolation, Purification and Characterization of an Extracellular Protease from a Locally Isolated *Bacillus sphaericus* SI-1

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ABSTRACT: An extracellular protease was purified from the culture supernatant of *Bacillus Sphaericus* strain SI-1 isolated from local soil sample. The enzyme purification was primarily performed by ammonium sulfate fractionation, centrifugation and dialysis followed by centricon filtration. In each step of processing, enzyme activity was assayed by azocasein digestion method. Partially purified enzyme was further purified by sephadex G-75 column chromatography. Fraction having protease activity was visualized on SDS-PAGE. The molecular weight of the protease was 41.70 KDa, while optimum pH and temperature were 8.4 and 38^{0}C respectively. The isolated protease is positively modulated by Ca++ and Mg++ ions but significantly inhibited by EDTA, Cu^{++}, Fe^{++} and Zn^{++} ions. The protease enzyme was slowly activated by sodium azide and inhibited by DTT and 2-marcaptoethanol. The enzyme activity could be completely eliminated by phenyl methyl sulfonyl fluoride (PMSF). Therefore, all the characteristics suggest the isolated protease to be a serine protease.

KEYWORDS: protease, protein, *Bacillus sphaericus*, phenyl methyl sulfonyl fluoride (PMSF).

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INTRODUCTION

Proteases are the class of enzymes responsible for the degradation of proteins to amino acids. They occupy unique position in health, medicine, industry and environment[1]. Proteases execute a large variety of functions and have important biotechnological applications[2]. Proteases represent one of the three largest groups of industrial enzymes that find application in detergents, leather, food, pharmaceutical industries and bioremediation processes[2-4]. For their diversified uses, these enzymes attracted the attention of physicians, scientists, industrialists and business personalities. Proteases are involved in the life cycle of various pathogenic organisms. As a result they have been made target for the design and development of drugs against pathogens. Human immunodeficiency virus (HIV) cannot be matured without the action of proteases on their structural proteins. Inhibition of these proteases may lead to death of the virus. Therefore, effort has been made to design inhibitors against the proteases which are essential for the maturation of structural proteins of HIV[5]. To the physicians proteases may represent the causative agent of diseases and the manufacturing industries a source of assistance or hindrance[1].

Proteases are one of the most important industrial enzymes produced by a wide range of microorganisms such as bacteria, yeasts, molds and also found in plants and various animal tissues. Bacterial proteases are mostly extracellular, easily produced in larger amounts, thermostable and active at wider pH range[6]. *Escherichia coli* synthesizes at least 25 different proteolytic enzymes which regulate the level of specific proteins and eliminate damaged or abnormal proteins[7].

Godfrey and West[8] stated that proteases account for 60% of the total commercial enzymes. Over the last decade commercial utility of these enzymes must have increased. Tannery wastes containing high amount of pollutants such as chromate, sulfide, emulsified fatty matters, colloidal proteins and waste limes liquor cause environmental pollutions[9]. Some tanneries have been forced to close down due to their pressure on environmental pollution[10]. Protease enzyme is an eco-friendly alternative of these chemicals. Extracellular alkaline proteases have been

introduced into the detergent industries more than fifty year ago, they currently occupy about 30 to 75 % of the detergent market in the advanced countries[11]. Alkaline proteases are very effective for the treatment of industrial effluents[12].

Therefore purification and characterization is the basic need to elucidate precise properties of an isolated enzyme for its application in industries. The purification process also increases the specific activities of enzymes, making them more targets oriented for industrial application[2]. Green and Hughes[13] salting out method for purification of proteins has been adapted for the preliminary purification and concentration of exo-proteases. Over the years other precipitants like metal salts, organic solvents, polyethylene glycols have been introduced for the purification of enzymes[14-16]. Ultra-filtration is an effective tool for clarifying, concentrating and purifying enzymes. It is specially used for the separation and concentration of extracellular enzymes[17]. Less expensive and easily applicable gel filtration or size exclusion chromatography for the separation and purification for proteins and enzymes is being used extensively[18-19]. Poly-acrylamide gel electrophoresis has been widely used to assess the purity of enzymes. A single band on the gel is evidence, though not proof of purity. SDS (sodium dodecyl sulfate) is commonly used to separate proteins having similar molecular weight[20].

In the current study, an extracellular protease from *Bacillus sphaericus* strain SI-1 was isolated, purified and characterized using various techniques along with methodologies mentioned above.

MATERIALS AND METHODS

Isolation, screening, and identification of protease producing bacteria

The microorganism used for the present investigation was isolated from environmental soil sample of Dhaka City in Bangladesh. Among the many strains which were grown on milk agar plate, the best protease producer was primarily selected on the basis of clear zone. Identification and characterization of the most potential bacterial strain was performed on the basis of different morphological and biochemical tests.

Protease enzyme production

The bacterium, *Bacillus sphaericus* strain SI-1 isolated from collected soil sample was aerobically cultured in an incubator shaker at 30°C and 180 rpm in sterile G media [solution 1(G1-g/l): $MgSO_4$ 0.2, KH_2PO_4 0.4, $MnSO_4$ 0.05, $ZnSO_4.7H_2O$ 0.005, $CuSO_4.5H_2O$ 0.005, $CaCl_2.2H_2O$ 0.005 and solution 2 (G2 g/l): K_2HPO_4 0.6, $FeSO_4.7H_2O$ 0.005, yeast extract 2.0, tryptone 10.0]. G1 and G2 media were autoclaved separately at 121°C temperature at 15 psi for 25 min and mixed just before inoculation in the ratio 1:19 respectively. The culture was incubated for 15-20 hours until the absorbance of the growing culture reached 1.5-1.8.

Extraction of protease from production media

The culture was centrifuged at 10,000 rpm in a Sorval RC-26 plus refrigerated super speed centrifuge for 10 min at 4°C. The supernatant was collected and used as the crude enzyme sample.

Enzyme assay

Protease activity was determined with azocasein as substrate by a modified method describe by Kreger and Lockwood[21]. Briefly, 800 µl of crude enzyme and 400 µl of 1.5% azocasein in 0.05 *M* Tris-HCl buffer (pH 8.5) were mixed and incubated in a water bath at 37°C for 30 min. the reaction was stopped by adding 2.8 ml of 5% TCA. After 15 min the solution was centrifuged at 4,000 rpm for 5 min. 2.0 mL of supernatant was then mixed with 2 ml of 0.5 *N* NaOH and absorbance was measured at 440 nm against appropriate blank. Protease activity was also assayed in subsequent steps of purification on the basis of this method.

Protein estimation

Protein content of crude extract as well as in different steps of purification process was determined according to the method described by Lowry *et al*[22]. One milligram of BSA dissolved in per mL distilled water was used as standard. The absorbance was measured at 650 nm in a SHIMADZU UV-160A spectrophotometer.

Purification of protease enzyme

The crude protein obtained from the bacterial extract was very dilute and it was concentrated by applying different methods which are described below.

Precipitation by ammonium sulfate and dialysis

Culture supernatant was taken in conical flask. Solid ammonium sulfate was added gradually with shaking to conical flask to make up to 75% saturation. The flasks were stored at 4°C for about 4 hours to complete precipitation. Protein precipitate thus obtained was separated by centrifugation at 10,000 rpm for 30 min. The supernatant was discarded and the pellets were dissolved in 1.5 ml of 0.05 *M* Tris-HCl buffer (pH 8.5) and stored at 4°C for further use. Salt containment of solution was removed by overnight dialysis in Tris-HCl buffer. After completion of dialysis, protein content and enzyme activity was measured as described earlier.

Concentration by centricon filtration

In the present work centriprep 30, 50 and 100 were used at their respective centrifugal force such as 1,500×g, 1,000×g and 500×g respectively for 15 min. Protease activity of each fraction was measured by azocasein digestion method. Target fraction was identified and subjected to further filtration in appropriate centriprep filter.

Sephadex G-75 column chromatography

Vertically placed 76 cm height and 2.5 cm diameter column was filled with appropriately swelled Sephadex G-75 beads in bubble free condition. Previously a piece of glass wool of appropriate size was placed at the bottom of the column using a glass rod. It was then allowed to stand for 2 hours. The column was then equilibrated with Tris-HCl-NaCl buffer (pH 8.0) under the same pressure used for packing the column. Homogeneity of the column was tested and void volume was determined. After application of sample and discarding the void volume, 81 fractions

each of 2.5 ml were collected in final round. The fractions were stored at 4°C. For each fraction the absorbance was taken at 280 nm to detect the presence of protein. The enzyme activity was determined by usual procedure of those fractions where protein was found.

Polyacrylamide gel electrophoresis
SDS-PAGE was performed on a slab gel containing 10% (w/v) poly-acrylamide by the method of Laemmli[20]. The sample along with standard markers was loaded in the well carefully avoiding cross contamination. The molecular weight was determined by interpolation of semi-logarithmic plot of relative molecular weight using standard molecular weight markers.

Characterization of purified protease

Optimum pH: For determination of the optimum pH of the enzyme, different buffers having different pH values were used. After pH adjustment of the buffer, azocasein was dissolved in different buffer solutions as substrate and enzyme assay was carried out within the pH range 4.0 to 11.0 (4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0, 10.0 and 11.0) at 37°C by azocasein digestion method.

Optimum temperature: Influence of temperature on protease activity was studied by incubating reaction mixture at different temperatures ranging from 15 to 70°C at an increment of 5°C intervals by using azocasein as substrate at pH 8.4.

Effect of metal ions and inhibitors: Effect of the different ions (Ca^{++}, Cu^{++}, Mg^{++}, Fe^{++} and Zn^{++}) on protease activity was studied by using varying the concentrations of respective ions in the range from 0.5 to 15.0 m*M* by adding them to a reaction mixture which was pre-incubated for 30 min at temperature 38°C and pH 8.4. The relative activities were estimated with reference to control. The activity of the protease was measured as described previously.

Effect of different inhibitors on protease activity: The protease solution was incubated at 38°C for 30 min and initial pH 8.4 with specific inhibitors. The residual activity was determined by azocasein digestion method. The inhibitors used were EDTA, PMSF, NaN_3, 2-mercaptoethanol and dithiothreitol. All the reagents were dissolved in deionized distilled water except PMSF which was dissolved in 75% isopropanol. Since the 2-mercaptoethanol was liquid, it was used as percent solution. The activity for 2-mercaptoethanol was tested in the range of 0.5 to 20.0%. The activities of the other inhibitors were tested from 0.5 to 10 m*M* concentrations.

RESULTS AND DISCUSSION

Bacillus sphaerious is a soil born spore former, an entomopathogenic bacterium. The aim of the present work is isolation, purification and characterization of extracellular protease from *Bacillus sphaericus* SI-1 isolated from soil of Dhaka, Bangladesh.

Isolated bacterium was grown in G media in laboratory scale to produce more enzyme sample, which also enhance more extracellular protease production. This protease along with other proteins was isolated by centrifugation at 10,000 rpm by discarding the cell debris.

The first step adopted in the purification of the enzyme was fractionation by ammonium Sulfate. Previous works reported that no significant amount of protein is precipitated, when the culture supernatant was saturated with ammonium sulfate up to 30%. When the salt concentration was reached to 40% enzyme activity was found in the precipitates. Enzyme activity increased with concentration of salt in the culture filtrate. No significant amount of proteins was precipitated after 75% of saturation. This means most of the proteins including enzyme present in the culture filtrate are precipitated when it is saturated to 75% with ammonium sulfate. Ammonium sulfate fractionation leads to 18.75 folds purification with respect to volume and 1.16-fold purification with respect specific activity. In this experiment high concentration of salt was used and dialysis was carried out at 0 to 4°C to remove salt to minimize denaturing of protease. Dialysis also helps to eliminate other small molecules along with salt.

The second step was purification of the sample by ultrafiltration with centricon. Ultrafiltration of protease though membrane is comparatively quick method. This method is used for partial purification of protein samples. It has fixed pore size, which allows defined molecular weight or less then it to pass through its filter. The result presented in the Table 1, clearly shows that protein having molecular weight less than 30 KDa and more than 100 KDa had very little activity. From this it may be concluded that our desired protein has molecular weight within 30 to 100 KDa. To narrow this range centricon 50 was used but it did not provide satisfactory result. The fractions having molecular weight 30-50 KDa show significant and 50-100 KDa show moderate enzyme activity. Filtration using centricons of different pore sizes gave maximum activity between 30 and 50 KDa.

Table 1. Comparative study of activity of protease after fractionation of protein based on molecular weight filter using centriprep.

Centricon	Molecular weight (KDa)	Total volume	Total activity (Unit)	Total protein (mg)	Specific activity (units/mg)
Centriprep 30	<30	34	3,400	12	283
	>30	46	74,333	78	952
Centriprep 50	<50	20	24,240	14.7	1,649
	>50	26	45,600	58	786
Centriprep 100	<100	15	26,000	19	1,364
	>100	11	12,600	32	393

Since the ultrafiltration study revealed that the potential protease might be between 30 to 50 KDa, it was subjected to gel filtration on sephadex G-75. The result presented in Figure 1 shows three distinct protein peaks. Only protein in the second peak shows protease activity while other two have no proteolytic activity. Protein contents of the fraction having proteolytic activity have been determined and the specific activity of the enzyme was determined. Spain AB *et al*[19] have reported the isolation, purification and characterization of a neutral protease from *Bacillus sphaericus* strain IT.

Gel filtration chromatography using sephadex G-75 has been successfully used to resolve the proteases fraction between 30 to 50KDa molecular weight. In this experiment 81 fractions were collected and monitored for the presence of proteins using UV spectrophotometer. Those fractions which absorbed at 28onm contained protein. Assay of enzyme activity revealed that protease was present only in second peak .This peak also has highest specific activity. The results have been presented in Fig.1.This data confirms that enzymatically active fraction was present in between 30 to 50KDa.

The result presented in Figure 2 on SDS-PAGE showed a single band in lane 1 and lane 3. Since only one band was clearly visualized after SDS-PAGE it may be assumed that the enzyme might be pure. SDS-PAGE electrophoresis failed to separate the protein into more than one band. This suggests that the protein might be single polypeptide chain. Molecular weight determination against standard markers suggests that the molecular weight of the protein might be 41.7 KDa.

The effects of pH and temperature on protease have been studied. The enzyme has pH optimum of 8.4 and it shows maximum activity at 38°C (Figure 3).

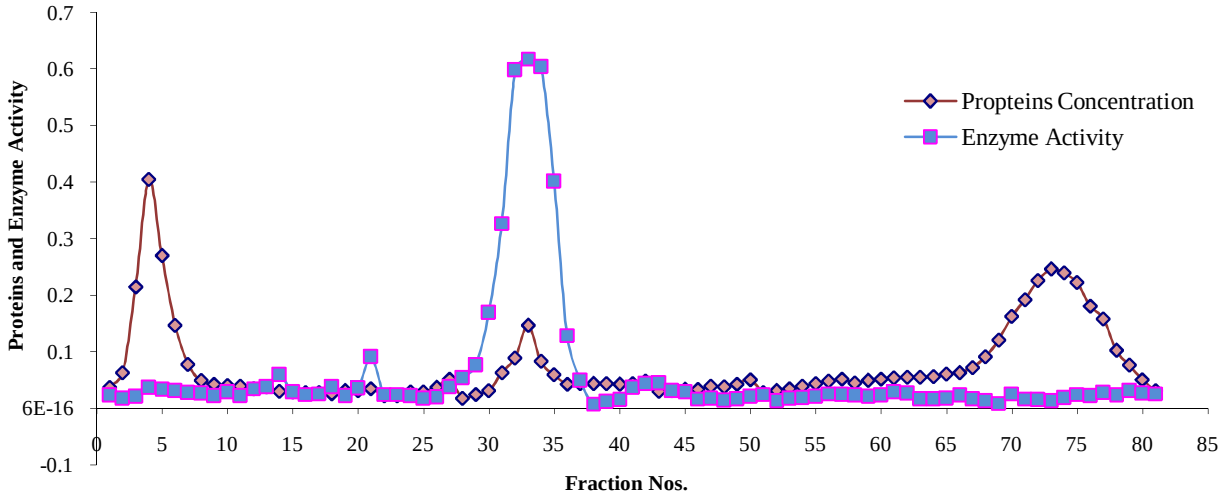

Figure 1. Fraction wise distribution proteins content and corresponding protease activity in gel filtration chromatography from fraction numbers 1 to 81 each of which 2.5 ml elution volume.

Extracellular protease isolated from *Bacillus sphaericus* SI-1 is positively modulated by low concentration of Ca^{++} and Mg^{++} ions, but at high concentration they negatively modulate the enzyme activity (Figure 4). This might be due to precipitation of enzyme at high concentration of these ions, because as ions corresponding salt was used which may be due to salting effect to precipitate. There is no significant difference on the activation of the enzyme by these ions. The metal may sit at sites other than the active site and can change the conformation of the protein, or it may bind near the active site. As a result it may bring some conformational change of the enzyme so that binding of the substrate to the enzyme facilitated.

Zn^{++}, Cu^{++} and Fe^{++} ions on the other hand inhabit the protease activity by about 75, 99 and 82% respectively at the highest (15.0 m*M*) concentration tested. It is also not studied whether the inhibition is competitive or non competitive. These divalent ions may occupy the same site as did the calcium and magnesium ions. These may also bind to the site other than that required by calcium and magnesium ions and bring about modification in the conformation of the enzyme so that the substrate no longer fit to the active site.

EDTA is a chelating agent of the metal ions. Addition of EDTA to the reaction mixture containing protease significantly (up to 75%) inhabits proteolytic activity (Figure 5). EDTA at a concentration of 2.0 m*M* is sufficient to eliminate 75% of the enzyme activity. No distinguishable decrease in activity was found after further addition of EDTA. This may be due to removal of metal ion from the reaction mixture.

A question naturally arises about the effect of EDTA and the divalent metal ions on the enzyme. The divalent cations may be the cofactor of the enzyme or not. If divalent cat ions are cofactors of the enzyme their removal

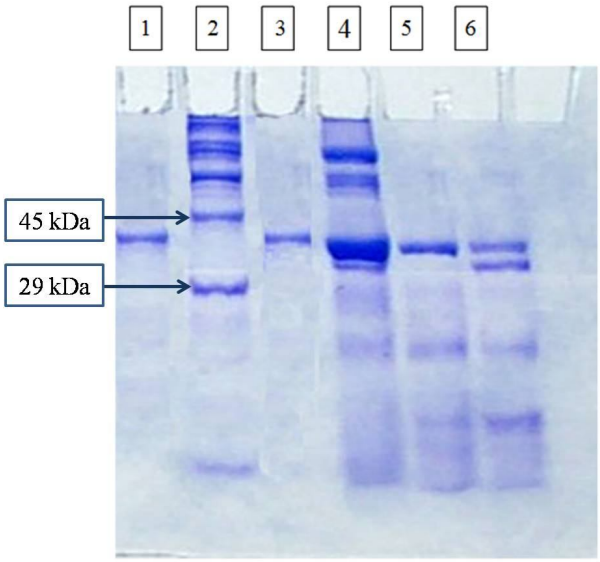

Figure 2. SDS-PAGE banding pattern, lane 1 and 3 - purified protein using sephadex G-75 column chromatography, lane 2 - marker proteins (from top 205 KDa, 116 KDa, 97.4 KDa, 66 KDa, 45 KDa, and 29 KDa), lane 4 - crude protein after ammonium sulfate fractionation and dialysis, and lane 5 and 6 -partial purified proteins of different batches.

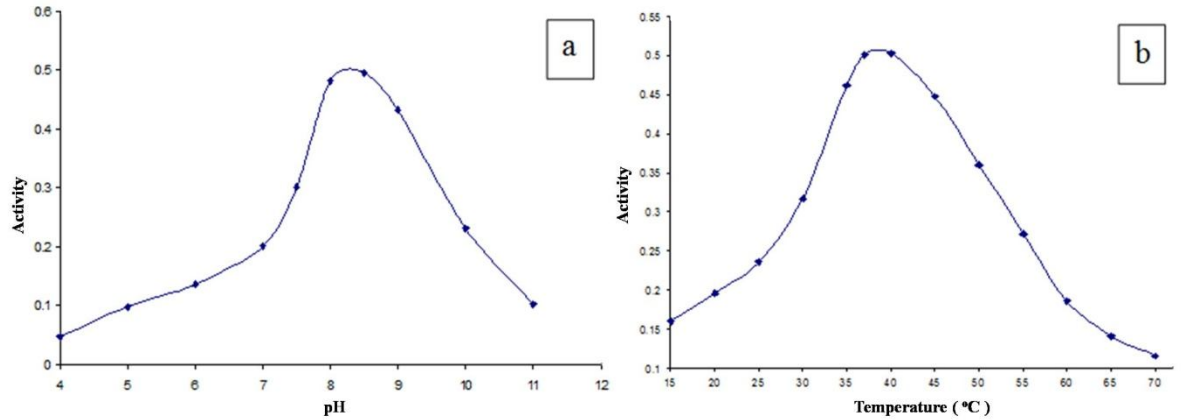

Figure 3. Determination of optimum pH and temperature for activity of the isolated protease. Activity was assayed (a) at different pH (4.0-11.0) and (b) at different temperature (15-70°C).

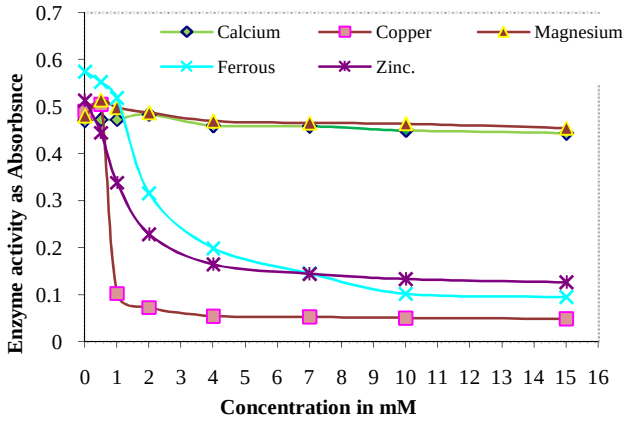

Figure 4. Effect of different divalent metal cations (Ca^{++}, Cu^{++}, Mg^{++}, Fe^{++} and Zn^{++}) each at different concentrations (0.0-15.0 m*M*) on protease activity.

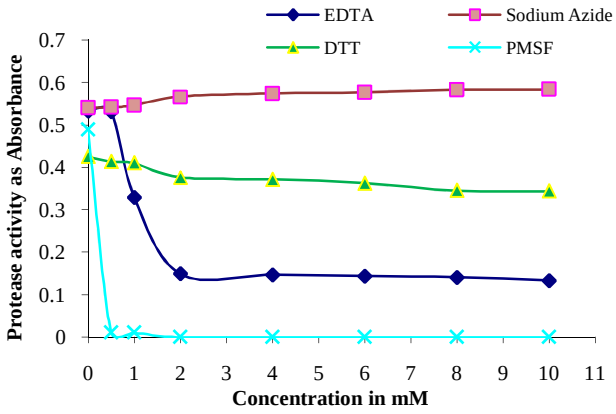

Figure 5. Effect of different modulators (EDTA, Sodium azide, DTT and PMSF) each at different concentrations (0.0-10.0 m*M*) on protease activity.

by EDTA should eliminate the total activity, but this was not found true for this enzyme. It may be due to the fact that metal ions bind at sites other than the active sites of the enzyme and activate it, and the removal of the metal ions by EDTA partially inhibits the enzyme. One-forth (25%) of the residual activity of the enzyme is not affected when the concentration of EDTA was raised from 2.0 to 10.0 m*M*. This suggests that the metal ions are required for partial activity of the enzyme.

Dithiothreitol and 2-mercaptoethanol are sulflyldral reagents. These reagents activate a protease if cysteine residue is present near the active site. The protease activity was progressively inhibited by the increasing the concentration of 2-mercaptoethanol in the reaction mixture (Figure 6). Since these reagents inhibit the enzyme, cysteine residue was not present at the active center. It might be that they have reacted with free sulfhyldal group at a site other than the active site and brought about a conformational change in the globular structure, which was required for proper enzyme activity and to activate enzyme. Sodium azide showed insignificant effect on the enzyme activity.

The most remarkable and distinguishable result was obtained by treating the enzyme with PMSF. Only 0.5 m*M* of the PMSF is sufficient to eliminate 98% of the protease activity. No activity was retained at 1.0 m*M*

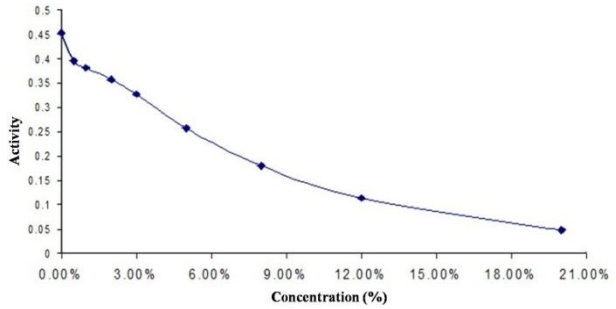

Figure 6. Effect of 2-mercaptoethanol concentrations on protease activity.

concentration of PMSF. PMSF is a serine protease inhibitor[2]. Sulfate group of PMSF form covalent adduct with serine group of the enzyme. This may protect substrate binding as well as catalysis and abolish total activity of the enzyme. If there were more than one enzyme such as metallo-protease, aspartate protease, etc. total elimination of enzyme activity could not be observed.

CONCLUSION

A protease was isolated, purified and characterized. Single band of 41.7 KDa in SDS-PAGE suggests that the protease might have been fully purified. Two ionizable amino acids are supposed to be the determining factors of the protease activity as revealed by bell-shaped curve one

of which is needed in protonated form and the other in the deprotonated form. Common cofactors were found to be slightly modulating the enzyme activity. Inhibition by DTT and 2-mercaptoethanol suggest the presence of sufhydryl bond in the enzyme. Inhibition by PMSF indicates the presence of a serine residue at the active site of the enzyme. Besides these, pH and temperature optima also suggest that the enzyme might be a serine protease. Further characterization of the purified protease is required for the assessment of its aptness as a commercial enzyme.

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