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Full Length Research Paper

Partial characterization and purification of protease enzyme from locally isolated soil bacterium

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ABSTRACT

The aim of this study to explore a novel protease enzyme was isolated from the local soil bacterium. The organism was gram positive and forms spore during adverse condition in the growth medium. After various tests it was suggested and the features agreed with the description of Bacillus subtilis in Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986). It was also identified as B. subtilis with 99.9% identity by API 50 CHB. The enzyme hydrolyses a number of proteins including Azocasein which suggests that it is an extracellular protease. Its optimum pH and temperature were 8.5 and 60°C. This microbial enzyme was partially purified by ammonium sulfate fractionation, dialysis, DEAE cellulose chromatography and electrophoretic analysis. Enzyme purity was tested by SDS-PAGE. It was found that a single band was appeared in the gel indicating the enzyme has separated and purified. Quantitative estimation has shown that 40mL of culture supernatant could dehair 2×1 cm of leather completely in 9 hours. In future the tanneries will use a combination of chemical and enzymatic processes. In practical applications, protease is a useful enzyme for promoting the hydrolysis of proteins and exhibiting remarkable dehairing capabilities

Keywords: Purification, SDS-PAGE, Characterization, Protease, Dehairing Enzyme, Isolation, Azocasein

INTRODUCTION

Leather industries are one of the most promising fields for export to earn foreign currency in Bangladesh. Most of the tannery industries in Bangladesh use chemicals for Dehairing that led great environmental and health problem. The tannery pollutants are causing heavily damage to water resources, agriculture, fisheries and finally to avoid the deleterious effects of chemical agents in tannery industries (Raju et al., 1996). Recently government of People's Republic of Bangladesh has taken initiative to develop the industry from outside the city and modernize it. Over the last few decades leather industry is based on large scale chemicals treatment which created worldwide environmental hazards.

In the back drop of this scenario enzymes started replacing poisonous chemicals from tannery industries. Enzymatic dehairing is suggested as an environment

Table 1. Different buffer used and their p^H ranges

Buffer	P^H range
Acetate buffer	4.0-5.6
Sodium phosphate buffer	5.6-8.0
Tris HCl buffer	7.5-8.9
Glycine-NaOH buffer	8.6-10.5

friendly alternative to the conventional chemical process (Nam and Pyun, 2002). The use of Proteolytic enzymes as an alternative to de-hairing skins has been investigated (Puvanakrishnan and Dhar, 1986).

Cleaner leather processing biotechnology has been used in the tanning industry for several years. At present, biological methods are being used with relative success in soaking, de-hairing, BATING and, in part, degreasing. Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides (Puvanakrishnan and Dhar, 1986). Proteases could play an important part in biotechnological applications like enzymatic improvement of feather meal and production of amino acids or peptides from high-molecular weight substrates or in the leather industry (Dhar and Sreenivasulu, 1984; Mukhopadyay and Chandra, 1993). In this report, the purification and characterization of a protease produced by this leather dehairing micro-organism is described.

MATERIALS AND METHODS

Reagents

Chemicals used in the experiment were from Oxoid Ltd. (Basingstoke, UK), Merck AG (Darmstadt, Germany), and Sigma (USA). Azokeratin was synthesized based on the method described in a previous study (Riffel, 2007).

Bacteria and growth conditions

Soil samples were collected from the poultry wastes in Savar, after serial dilution, culture was given in LB broth media from the sample for 16 h at 37°C. At the next day single colony was found. Among them few colonies were identified on the basis of different colony morphology. Each colony was inoculated into screw capped test tubes containing autoclaved feather with liquid broth media and incubated overnight at 37°C with shaking at 160 rpm. One media was used as negative control.

Identification of the Selected Isolate

In order to identify the selected isolate, the following characteristics were studies according to Bergey's Manual of systematic Bacteriology. Microscopic examination of the bacteria employed the Gram staining and Spore staining of the bacteria. Several biochemical tests were performed for the identification of selected bacterial isolate. These tests include Carbohydrates Fermentation, Catalase, Hydrolysis of starch, Methyl Red-Voges Proskaure, Indole Production and Nitrate Reduction Tests.

Protease production and determination of its proteolytic activity

The microorganism was cultivated in sterile nutrient broth medium. The culture was grown overnight on a rotary shaker at 150 rpm and incubated at 37°C for 15-20 hours. The culture was then centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was collected and used as crude enzyme sample. Proteolytic activities were assayed by Azocasein test, described by Kreger and Lockwood (1981) was done. Here azocasein is used as a substrate. Optical density was measured at 440 nm.

Effect of pH and temperature on enzyme activity and stability

For determining the effect of pH on protease activity different buffer system with different pH were used. Azocasein was dissolved in different buffer solution and the enzyme assay was carried out within pH range (4.0 to 10.5) by azocasein assay method. All of them were used at 0.05M concentration. Table 1 above.

For the determination of the effect of temperature, the reaction medium was incubated at varied temperature and the protease activity was determined. For this purpose the enzyme preparation was added to a mixture of 1 mg 1 % azocasein solution, 0.1 ml of 0.06 M CaCl2 and buffer (0.2 M Tris-HCI buffer, pH 8.0) and incubated at 37°, 40°, 50°,60°,65°C temperatures.

Effects of protease inhibitors, reducing agents, and metal ions on keratinase activity

The activity of the isolated protease was tested in the

presence of various known protease effectors, EDTA, 2-mercaptoethanol, potassium di-chromate, sodium thiosulfate. The azocasein assay was used with the addition of these effectors solution to achieve a final desired effectors concentration of 5mM. Control was taken where azocasein assay without these effectors was carried out. The protease activity was measured with adding different salts like $ZnSO_4$, $MgSO_4$, $CuSO_4$, NaCl, KCl at different concentration and then azocasein assay was performed.

Observation of dehairing capability of the enzyme

For dehairing studies, the organism was grown in nutrient broth at 37ºC for around 20 hours. Then it was centrifuged at 4000 rpm for 8 minutes. The cell free supernatant was added on detergent washed goat skin to observed enzymatic dehairing capability of the organism. Sodium azide was used at 1% so that no organism can grow. Nutrient broth was used as control.

Determination of ammonium sulfate fractionation and dialysis of protein mixtures

All subsequent purification steps were carried out at 0–4 °C.
For Ammonium sulfate fraction of protein mixtures, 20 hours grown bacterial culture was centrifuged at 8000 rpm for 6 minutes. The cell free supernatant was then saturation with ammonium sulfate slowly but frequently to dissolve in crude culture supernatant. After 60% saturation culture supernatant was kept in freeze for 12 hours. After that time maximum protein was precipitated. Then centrifuged at 14000 rpm for 7-10 minutes to collect the precipitates and precipitates were redissolved in Trise-HCl (0.1M, pH 7.7) buffer. Dialysis was carried out to remove the ammonium salts in a cellophane bag for 8-12 hours using Trise-HCl buffer. Then the collected sample was stored at -20^{o}C for chromatographic analysis.

Ultra filtration by millipore centricons

Ultra filtration using centricons was used to separate the proteins having molecular weight around 100 KDa. The protein having molecular weight 100 KDa or above were retained in the upper part of the centricon while small proteins were passed through the membrane filtrate after centrifugation for 30 minutes at 5000 rpm in a Sorval super speed centrifuge.

Ion-Exchange column chromatography for protein purification

The enzyme solution was applied to a DEAE cellulose powder in 0.1M Trise-HCl buffer (pH 7.5) in a beaker and left it to swell for few hours. The gel suspension was packed in a column of desired length. After packing the column was equilibrated with 0.1M Trise-HCl buffer. The proteins were eluted according to their molecular weight from the column with same buffer by linear and the adsorbed proteins were then eluted with a linear gradient of 0.1–5.0 M NaCl in the same buffer at a flow rate of 12 ml/ min). The absorbance was taken at 280nm to measure the OD of collected fractions (200 tubes). The most active fractions were concentrated from 15ml to 3ml by PEG-6000. Then the concentrated sample was stored at -20^{o}C for gel analysis.

Examination of Enzyme Purity

As described by Laemmli (1970), the protein purity of the enzyme was evaluated by SDS-PAGE using 1 mm thick slab gels containing 14% (w/v) separating gels and 5% (w/v) stacking gels. After running the gel was fixed overnight in a solution of TCA and stained with Coomassie brilliant blue G-250 using the ultrasensitive method. Alternatively the gels were submitted to silver staining. It was then kept immersed in freshly prepared destaining solution till the gel background became transparent. The electrophoretic migration of the protein was compared with that of low-molecular-mass protein markers (Pharmacia, Sweden). Zymography was determined according to the method described in the study of Riffel et al. (2007)

RESULTS

Characterization of the Isolated Soil Bacterium

The main object of this work was to isolate and characterize thermophilic enzyme which could specifically be used for dehairing the hides and skins of cattle in the tannery industries. In this connection three ways were planned. One was to isolate thermophilic organism from different natural sources. The others is to characterize and identification of the isolated organism. The growth phenotype and some of the biochemical characteristics of the organism was determined. This organism was characterized and identified as a member of gram positive Bacillus family by several test. The features agreed with the description of *Bacillus subtilis* in Bergey's

Table 2. Morphological and biochemical test for the characterization of B. subtilis

Test performed	Observations	Results
Streak plate isolation:		
NA at 37°C	milky colonies	Positive
Gram stain	Small violate colonies singly	Gram positive rods
Spore stain	green color appeared	Spore forms
Cultural characteristics:		
Catalase Test	bubbles formed	Positive
Indole (SIM)Test	bright red ring, growth away	Negative
Nitrate Reduction Test	no color change after zinc dust addition	Positive
Urease Test	no bright pink color	Negative
Methyle Red Test	deep red ring formed	Positive
Sucrose Fermentation	yellow	Negative
Gelatin Hydrolysis	remain liquefied at 4º	Positive
Voges Proskauer Test	weak red ring formed	Positive
Starch Hydrolysis	bright zone	Positive
Glucose	Yellow color	Positive for acid and negative for gas
Citrate test	change in color	Positive for citrate utilization

Figure1. Azocasein test; where sample on right and control on left.

Table 3. Effect of pH on Protease Activity

pH	Activity of Enzyme(unit)
4.0	28
5.0	36.5
6.0	48
7.0	63
8.0	68
8.5	70
9.0	66
10	60
11	48

Manual of Systematic Bacteriology (Sneath et al., 1986). It was also identified as *B. subtilis* with 99.9% identity by API 50 CHB. So this bacteria is named here as a Bacillus *subtilis.* The results are presented here. Table 2.

Assay for proteolytic activity of the enzyme

Proteolytic activities were assayed by Azocasein test, described by Kreger and Lockwood (1981) was done. Here azocasein is used as a substrate. The proteolytic activity was found as 21.13 units for the sample. One unit of proteolytic activity is defined as the amount of enzyme that produces an increase in the absorbance of 0.01 at 440nm. Figure 1.

Effect of p^H on Protease Activity from the Organism

The pH of the reaction media can affect the protease activity. For this purpose the enzyme activity over a pH range between 4 and 11 was studied. Table 3.

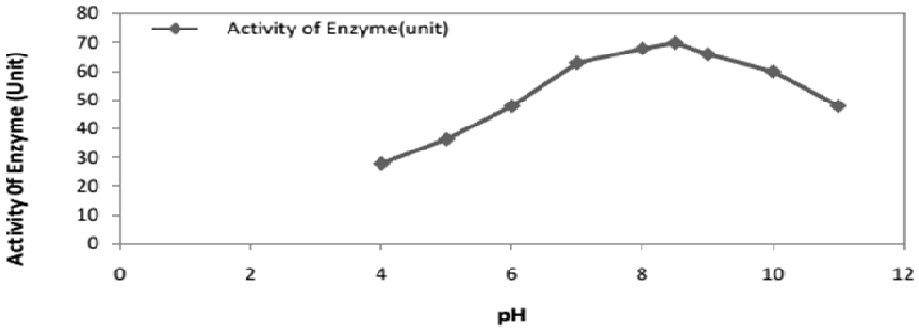

Figure 2. Graphical presentation of effect of pH on protease activity

Table 4. Protease activity at different temperature (by Kreger and Lockwood method)

Temperature	Absorbance at 440nm
0°C	0.009
4°C	0.019
20°C	0.121
30°C	0.181
37°C	0.183
40°C	0.191
50°C	0.205
60°C	**0.250**
65°C	0.105
80°C	0.019

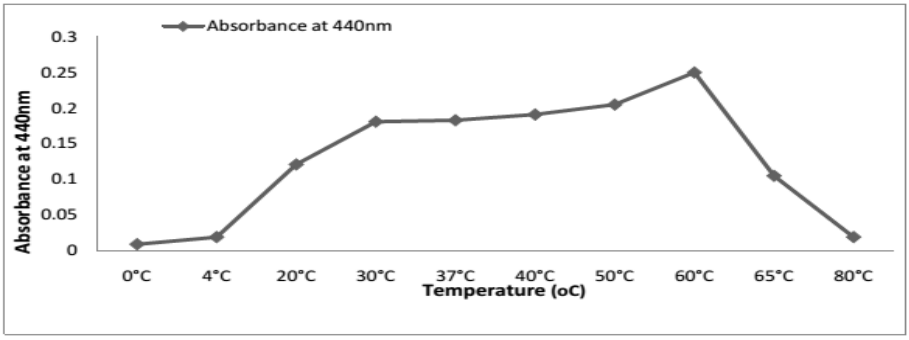

Figure 3. Graphical presentation of protease activities at different temperature

The enzyme shows its maximum activity at pH 8.5. The activity decline at pH 8.0 or above 8.5. Therefore pH 8.5 might be the optimum pH for enzyme activity. Additionally, its optimum pH was similar to that of previous reports (Scopes, 1982). Most proteases are active in neutral to alkali conditions, from pH 7.0 to pH 9.5. For example, the activity optimum of protease from *Mycobacterium* kr10 is pH 7.0 (Tsujibo and Inamori, 1990), *B. pumilus* FH9 of pH 8.0 (Gessesse et al., 2003), *Fervidobacterium islandicum* AW-1 of pH 9.0 (Puvanakrishnan and Dhar, 1986).

The figure 2 shows that the enzyme activity increase with the increase of pH of the media and the optimum pH is 8.5 for the activity of protease enzyme in Tris-HCL buffers. The results showed that the optimum pH of the protease enzyme was 8.5. Studies on growth temperature and pH suggest that the organism might be alkaline and thermophilic Bacillus

Effect of temperature on enzyme activity

The activity of the enzyme was measured over a range of temperature (0°C, 4°C, 20°C, 30ºC, 37ºC, 40ºC, 50ºC, 60ºC, 65ºC, 80°C) and the result is presented in Table 4 and Figure 3.

Table 5. Effects of salts and other chemicals on the activity of the protease

Compound (concentration in mM)	Caseinolytic activity (%)
Control	100
$MgSO_4$ (5)	109
$ZnSO_4$ (5)	77.2
EDTA (5)	92.5
EDTA (5) + $ZnSO_4$ (5)	82.5
EDTA (5) + $MgSO_4$ (5)	102
EDTA (5) + $CuSO_4$ (5)	84.2
NaCl(100)	100
NaCl(200)	100
β-Mercaptoethanol(5)	44.9
Sodium thiosulfate(5)	78.9
Potassium per manganate(5)	96.7

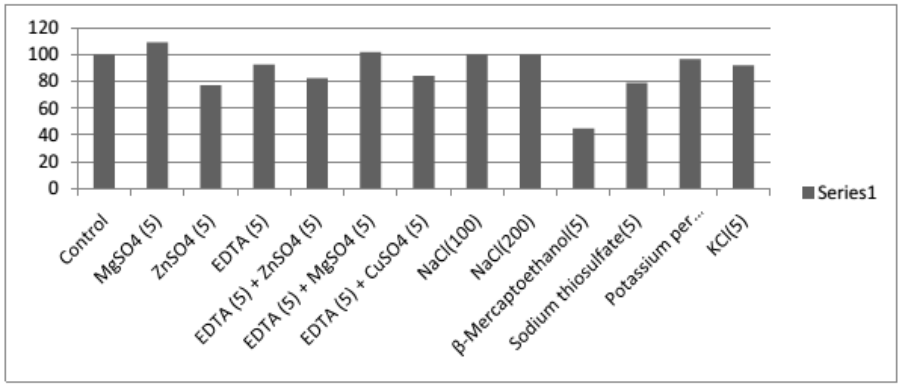

Figure 4. Graphical presentation of effects of salts and other chemicals on the activity of the protease

The enzyme activity is increased with the increase of temperature. The experiment was reported 2 times and the result is reproducible. There was a significant increase in enzyme activity between 20°C to 55°C. The enzyme seems to be active at 60°C and its activity declines as the temperature increase beyond 60°c. At 80°c the enzyme has very little activity. This suggests that the enzyme might be a thermostable enzyme. Figure 1 shows that the protease was active over a temperature range of 4°C ~80 °C, with an optimum at 60°C. Most proteases possess an activity optimum in the range of 30~80 °C, for example, protease from *B. pseudofirmus* AL-89 is of 60~70 °C (Ghorbel-Frikha and Nasri, 2005) and a few have exceptionally high temperature optimum of 100 °C (Puvanakrishnan and Dhar, 1986).

Effect of salts and other effectors on the protease activity

The effect of different salts ($MgSO_4$, $ZnSO_4$, $CuSO_4$, NaCl, KCl) and other effectors (EDTA, 2-mercaptoethanol, sodium thiosulfate) at different concentration was measured. $MgSO_4$ increased the activity and β-Mercaptoethanol decreased the activity of the enzyme. NaCl didn't change the activity. Others had little deactivating effect. Table 5 and Figure 4.

The result shows that 5mM Mg^{++} ion slightly increased the activity of the enzyme while Zn^{++} showed slightly decrease. Other elements Na^{+}, K^{+} had no effect on the enzyme. EDTA showed no effect on the protease activity which suggested that the enzyme might not be metallo protease. The enzyme activity was significantly reduced by β- Merceptoethanol. β-Mercaptoethanol has been reported to stabilize cyste in proteases by protecting the oxidation of sufhydral group in proteins (Sneath et al., 1986). No effect of EDTA was detected on enzyme activity suggesting that the metal might not be involved in enzyme activity (Moallaei and Bouchara, 2006).

Observation dehairing capability of the isolated protease enzyme

The cell-free supernatants were used as sources of crude enzyme. The treated skins and controls showed visible

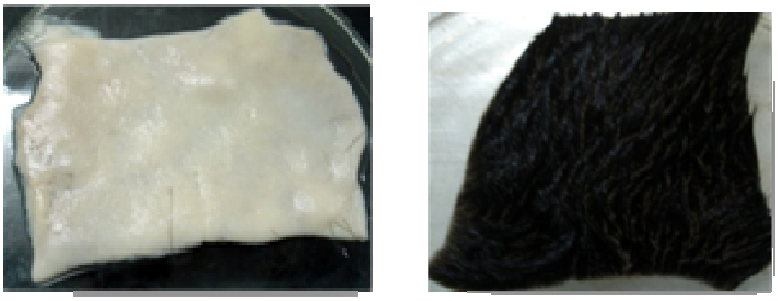

Figure 5. Enzymatic dehairing by B. subtilis protease on left, control is on the right (without protease)

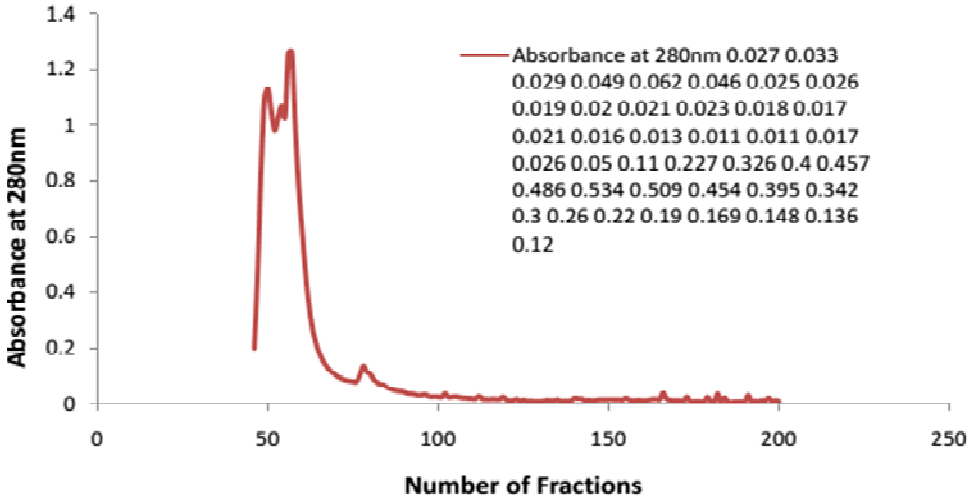

Figure 6. Graphical presentation of OD of collected fractions from DEAE cellulose column chromatography

differences after 9 h incubation. No color alteration was observed, although the presence of depilated areas was noticed in the skins treated with enzymes. When hairs were pulled with a forceps, they were very easily released after enzyme treatment.

After 9 h incubation intact hairs could be taken out of the skins easily by simple scraping. In controls, hair loosening was not observed, even by the mechanical action of a forceps. This result was much better than other different bacteria that also caused dehairing. Proteases have been used in the hide dehairing process, where dehairing is carried out at pH values between 8 - 10 (Kembhavi et al., 1993). Figure 5.

Partial purification of protease enzyme

To remove unwanted proteins from the crude enzyme solution, 40–80% saturation of $(NH_4)_2SO_4$ had the best effect on enzyme purification. Most of the protein in bacterial culture filtrate precipitated at 60% saturation. The obtained most active enzyme protein preparation could be obtained at the ammonium sulphate level of 60 %. This result was in complete accordance with other workers (Kojima et al., 2006; Nam and Pyun, 2002).

The overall purification factor was about 22.6 fold and the final yield was 51%. The final product had a specific activity of about 839.41 U/mg. Protein purification and different enzymetic properties of the protease are presented in a table 6. Ion-exchange DEAE cellulose column chromatography was for protein purification. The desired enzyme was found in 53-55 numbers tube by Azocasein test. The result is presented in figure 6.

Figure 6 shows that the desired enzyme was found in 53-55 numbers of tubes/fractions and it was also found that 54 numbers of tube/fraction contains large amount of desired enzyme.

Enzyme purity was tested by SDS-PAGE according to Laemmli (1970) and operated at 4°C. It was found that a single band is appeared in the gel. It proves that the

Table 6. Protein purification and different enzymatic properties of the protease

Protein purefication status	Protein concentration (mg/mL)	Protease activity	Enzyme unites/mL	Specific activity	Protein purification fold
Crude culture supernatant	0.82	1.159	289.75	350	1
60% saturation with ammonium sulfate	3.52	1.79	447.5	127	2.3
Removal of salt by dialysis	1.96	0.967	242	123	2.9
Ultrafiltration by centricon	1.36	1.01	252.5	185	4.9
Gell filtration chromatography	135µg/mL	0.217	54.25	54.41	11.5

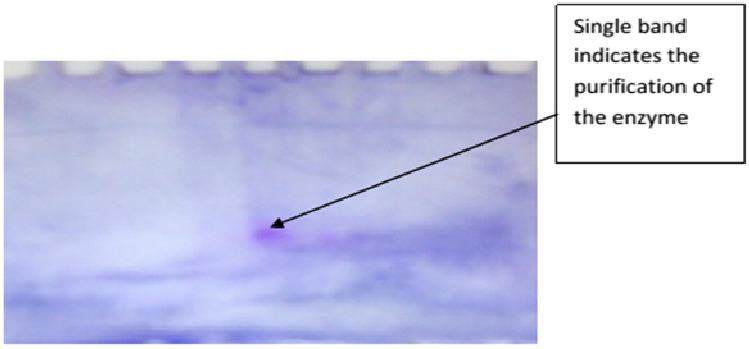

Figure 7. SDS-PAGE of protease obtained from Bacillus sp.

enzyme has purified and separated.

The figure 7 represent a single band indicating that the enzyme has purified and further test can be done to identify the molecular mass and other characterization of the protein.

DISCUSSION

Novel protease enzyme was isolated from local soil bacterium showing remarkable de-hairing activity of cow hides and skins both qualitatively and quantitatively. After various biochemical characteristics, morphological tests it was suggested and the features agreed with the description of *B. subtilis* in Bergey's Manual of Systematic Bacteriology (Soares et al., 2005).

Azocasein assay developed by Kreger and Lockout is a well accepted method for the assay of wide variety of protease having overlapping specificity. The enzyme hydrolyses a number of proteins including Azocasein which suggest that it is an extracellular protease (Dhar and Sreenivasulu, 1984). Bacillus species have been reported to produce proteases (Macedo and Henriques, 2005; Tatineni and Mangamoori, 2007). Therefore, it may be called a very good method for the large scale screening of bacterial protease (Ishikawa and Fujiwara, 1993).

The enzyme seems to have an optimum temperature of 60ºC. Most proteases possess an activity optimum in the range of 30~80 °C, for example, protease from *B. pseudofirmus* AL-89 is of 60~70 °C (Ghorbel-Frikha and Nasri, 2005), *Nocardiopsis* sp. TOA-1 is of 60 °C and a few have exceptionally high temperature optimum of 100 °C (Puvanakrishnan and Dhar, 1986). The enzyme seems to have an optimum pH of 8.5. Additionally, its optimum pH was similar to that of previous reports (Rozs et al., 2001). *B. subtilis* strains had been widely utilized for enzyme production, including the proteases (Macedo and Henriques, 2005; Madern and Zaccai, 2000).

The effect of a number of ions on the activity of the enzyme was observed. Mg^{++} at 5-10mM level slightly enhances the enzyme activity while Zn^{++} ions slightly decrease the activity of the enzyme. β- Mercepto ethanol is an inhibitor of protease. β-Mercaptoethanol has been reported to stabilize cystein proteases by protecting the oxidation of sufhydral group in proteins (Sneath et al., 1986). No effect of EDTA was detected on enzyme activity suggesting that the metal might not be involved in enzyme activity (Moallaei and Bouchara, 2006).

Enzymatic de-hairing may be the ideal process. Quantitative estimation has shown that 40mL of culture supernatant could dehair 2×1 cm of leather completely in a 9 hours. After 9h incubation intact hairs could be taken out of the skins easily by simple scraping. This shows that the bacterial isolate moderate to high amount of enzyme for dehairing. Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides (Riffel, 2007). A significant feature of the enzymatic de-hairing process is complete hair removal and minimal usage of sulfide and the decomposition products formed from the

tannery wastewater, with great improvement in wastewater quality as a result.

A trial was given to obtain the partially purified proteases from the culture supernatant of *Bacillus sp.* from one hand to create an interesting comparative study of the characteristics of the purified enzyme preparations from the other hand. This microbial enzyme was partially purified by ammonium sulphate fractionation, dialysis, DEAE cellulose chromatography and electrophoretic analysis. The obtained most active enzyme protein preparation could be obtained at the ammonium sulphate level of 60%. This result was complete accordance with other workers (Kojima et al., 2006; Nam and Pyun, 2002). The protease precipitated by the ammonium sulphate had been reported in many previous studies (Tsujibo and Inamori, 1990; Thys, 2006). The precipitates were found to be very active after the dialysis. This gave 2.9 fold purification of the proteins. Ultra filtration is another method for the separation of proteins of different molecular weight (Suntornsuk and Oda, 2005). Proteins having molecular weight higher than or equal to 100kDa were used. In this process the protein were purified to 4.9 fold.

After ultra filtration protein was further purified by gel filtration chromatography using DEAE cellulose. This method is very laborious and time consuming but separation of protein is very reliable. Three different protein picks of different molecular weight was found and one of the pick showed considerable enzyme activity (Cappuccino and Sherman, 2001; Hartree, 1972; Sookkheo and Chen, 2000). In this process the protein was purified to 11.5 fold. The subunit molecular mass of the protease was estimated by comparing the electrophoretic mobility of the protease with the electrophoretic mobilities of marker proteins. It was found that a single band was appeared in the gel indicating the enzyme has fully separated and purified. The level of purification is higher than those reported in other similar papers (Kim, 2004; Mukhopadyay and Chandra, 1993; Nam and Pyun, 2002).

CONCLUSION

A new protease produced by Bacillus sp. was partially purified and characterized. The results showed that the *B. subtilis* proteases enzyme can be utilized in enzymatic dehairing of cow skin in tannery industry to control the environment from pollution, which is a prerequisite for biotechnological applications. The culture characteristics and biochemical tests of the organism suggest that it is a thermophilic, Gram positive, spore forming and aerobic bacteria. The characterization of protease so far showed that it is an alkaline protease, highly active at temperature near 60ºC. As the bacterial protease showed high activity in dehairing of cow skin and our next target is to introduce it to the tannery industries, so that they can use it instead of hazardous chemicals for better leather quality and most importantly for a better environment.

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