

Purification and characterization of protease from mangroves derived strain of *Bacillus cereus*

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Abstract: Protease enzymes of most potent strain, *Bacillus cereus*, were purified in 2-step procedures involving ammonium sulfate precipitation, sephadex G-200 and sephadex G-100 gel permeation chromatography. The purification steps of proteases specific activities of 727.4 u/mg protein/ml. The maximum protease activities for enzymes were attained at 30°C, pH 7.2, 1% of chitin concentration and 0.4 enzyme concentrations. The obtained data showed that the purified proteases were different enzymes not identical ones. It was suggested that applications of protease of the present purified enzymes in the fields of pharmacy, chemotherapeutic agents and/or industrial purposes and this suggestion, however, needs further investigation.

INTRODUCTION

Proteases execute a large variety of functions and have important biotechnological applications. Proteases represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes (Anwar and Saleemuddin, 1998; Gupta *et al.*, 2002). Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Anwar and Saleemuddin, 1998; Beg and Gupta, 2003). *Bacillus* produces a wide variety of extra-cellular enzymes, including proteases. Several *Bacillus* species involved in protease production are e.g. *B. cereus*, *B. stercorophilus*, *B. mojavensis*, *B. megaterium* and *B. subtilis* (Ammar *et al.*, 1991; Sookkheo *et al.*, 2000; Beg and Gupta, 2003; Banik and Prakash, 2004; Greze *et al.*, 2005 and Soares *et al.*, 2005). Probably the largest application of proteases is in laundry detergents, where they help removing protein based stains from clothing (Banerjee *et al.* 1999; Banik and Prakash, 2004). For an enzyme to be used as a detergent additive it should be stable and active in the presence of typical detergent ingredients, such as surfactants,

builders, bleaching agents, bleach activators, fillers, fabric softeners and various other formulation aids. In textile industry, proteases may also be used to remove the stiff and dull gum layer of sericine from the raw silk fiber to achieve improved luster and softness. Protease treatments can modify the surface of wool and silk fibers to provide new and unique finishes. Proteases have been used in the hiddehairing process, where dehairing is carried out at pH values between 8 and 10 (Ishikawa *et al.*, 1993). Proteases are also useful and important components in biopharmaceutical products such as contact-lens enzyme cleaners and enzymatic debriders (Anwar and Saleemuddin, 2000). The proteolytic enzymes also offer a gentle and selective debridement, supporting the natural healing process in the successful local management of skin ulcerations by the efficient removal of the necrotic material (Sjodahl *et al.*, 2002).

In an earlier study we have isolated *Bacillus cereus* (Mahmoud *et al.*, 2007) which considered as most potent protease producer. With respect to the factors affecting productivity and properties of this protease, it was considered of interest to purify this enzyme and investigate the factors affecting their activity. In this paper we aimed to purify protease and to study the factors affecting the activity to present potential application of the protease for industrial proposes.

MATERIAL AND METHODS

Bacterial strain

The strain *Bacillus cereus*, isolated from mangroves rhisophere soil and showing a high proteolytic activity, was used in this study. Therefore, *Bacillus cereus* was selected to purify its extracellular protease and to characterize the optimal condition affecting on activity of pure enzyme.

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Purification of protease

The purification of protease obtained from *bacillus cereus* strain was done according to Ammar *et al.* (1991). The enzyme was purified in a 2-step procedures involving ammonium sulphate precipitation, and Sephadex G-200 & G-100 gel permeation chromatography.

Production of protease

Five ml of overnight culture of *bacillus cereus* strain was used to inoculate 250 ml Erlenmeyer flask containing 50 ml aliquots of liquid production medium [consisted of (g/l, w/v): Chitin, 10.0; yeast extract, 1.0; KH_2PO_4 , NH_4HPO_4 , CaCl_2 , 0.1; $\text{MgSO}_4 \cdot 5\text{H}_2\text{O}$, 0.05]. The inoculated flask was incubated at 30°C for 18 h. An additional sub-culture was made by transferring 2.5 ml of 18 h culture into 2-liter flask containing 1-liter of liquid production medium and incubated at the same condition. The bacterial cells were harvested by centrifugation at 15000 rpm for 10 min at 4 °C. The obtained supernatant was filtrated using a Millex®-GS 0.22 µm filter (Corrigtwhill, Ireland) and the supernatant was used as the starting point for purification as follow

Dialysis of crude enzyme filtrate against sucrose

The supernatant was transferred into a special clean plastic bag for dialysis which was previously cut into the desired length, with some extra-length for the knots. Two knots were tied at one end, closed together, and then filled with the enzyme filtrate, then the filled sac was covered with sucrose in a glass jar, the external sucrose solution should be changed every few hours during the dialysis process. After dialysis was completed, the sac was emptied into a beaker by puncturing it carefully. The plastic bag was transferred into a new jar filled with distilled water, the plastic bag must be completely covered with distilled water to avoid any shrinkage and subsequently losses its permeability. The external distilled water should be changed every four hours during the dialysis process until the enzyme solution volume inside the plastic bag reaches a constant one.

Ammonium sulphate fractionation

The dialyzed cell-free filtrate was first brought to 20% saturation by the gradual addition of solid ammonium sulphate while stirring at 4°C, after 30 min period of equilibration, the precipitated protein was obtained by centrifugation for 15 minutes at 15000 rpm at 4°C. The obtained pellet was re-suspended in a 10 ml of ice-cold 0.2M phosphate buffer pH7.2. Centrifugation was repeated and the pellet was re-suspended in order to determine both the enzyme activity and the protein content whereas the left supernatant was supplied again with ammonium sulphate in order to achieve 40, 60, 80 and 100% saturation of ammonium sulphate precipitation. The enzyme activity and protein content were determined for each separate fraction (Lowery *et al.*, 1951; Ammar *et al.*, 1991).

Dialysis against phosphate buffer

The procedure was performed as previously mentioned with repeating the dialyzation step against distilled water followed by dialysis against phosphate buffer pH7.2. The obtained enzyme, however, was concentrated against sucrose

to be available or the application on column chromatographic technique.

Fractionation pattern by applying column chromatographic technique

The enzyme obtained from the above step was loaded onto a Sephadex G-200 column (Pharmacia 2.6 x 70 cm), pre-equilibrated with 0.2 M phosphate buffer pH 7.2, and then eluted with the same buffer. Preparation of the gel column and the fractionation procedure was carried out as mentioned by Abdel-Raouf (1990). Fifty fractions were collected (each of 5ml). Sharp peaks of fraction obtained after applying sephadex G-200 column were collected and concentrated using a new plastic bag for dialysis. The enzyme obtained from the Sephadex-G200 column was loaded onto a second column (Pharmacia 2.6x70 cm) packed with sephadex G-100. Fifty fractions (each of 5ml) were collected and both the enzyme activity as well as the protein content was performed for each separate fraction.

Factors affecting activity

The following factors were studied to obtain the optimal condition for activity of purified proteases. These factors include incubation period, incubation temperature, pH and concentration of enzyme.

Temperature

This experiment was carried out in order to determine the effect of different incubation temperatures of the reaction mixture on the purified protease enzymes activities. This was performed by incubating the enzyme reaction mixtures at different temperatures viz: 10, 20, 30, 35, 40, 50 and 55°C respectively. The assay of protease activity was performed as previously mentioned.

pH value

This experiment was performed in order to investigate the effect of different pH values on the purified protease enzyme activities. The purified enzyme reaction mixture was incubated at different pH values via: 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8 and 8.0 using 0.2M phosphate buffer. The enzyme activities for each case were determined.

Substrate concentration

This experiment was undertaken to investigate the effect of different substrate concentrations on the activity of the purified protease. Chitin was applied at the concentrations viz. (W/V, %) 0.25, 0.5, 1.0, 2.0, 5.0 and 10.0 respectively. enzyme activities were determined

Enzyme concentration

This experiment was carried out in order to determine the effect of enzyme protease concentrations on the purified enzyme activity. Serial dilutions were performed for each of the four isolated purified enzymes. The dilutions in terms of mg protein/ml, for protease produced *bacillus cereus* MAB5 (HQ675025): 0.003, 0.006, 0.0125, 0.0250, 0.050, 0.10, 0.20 and 0.4 were prepared. The enzyme activity was determined as mentioned before.

Assay of proteolytic activity

Determination of the activity of purified protease was done according to chitin clearing zone technique described by Ammar *et al.*, (1991). Briefly, 250 µl of the crude or purified enzyme were introduced into each one of three cups of protease assay plate which, consists of liquid production medium in addition to agar 15 g/l. The plates were incubated for 8 hrs at 30 °C, the remainder supernatant was removed and the plate was flooded with 10 ml of the mercuric chloride solution. The resulting diameters of the clearing zones were recorded and then taken as indications of proteolytic activities and compare with standard curve for protease from Sigma (Mahmoud *et al.*, 2007). The method of Lowry was used for protein quantitation with a bovine serum albumin standard curve (Lowry *et al.*, 1951).

RESULTS

Production and purification of protease of *bacillus cereus* MAB5(HQ675025)

One liter of the bacterial broth was centrifuged at 12000 rpm for 15 min at 4°C. The collected supernatant (910 ml) containing the active protease enzyme was filtered through 0.22 µm filter under aseptic conditions. Proteolytic activity of crude protease enzyme was 517.8 u/mg of protein/ml.

Dialysis of enzyme

The obtained filtrate (910 ml) was then dialyzed using special plastic bags for dialysis against pure sucrose crystals. A final volume obtained from dialysis was 65 ml; therefore the concentration of the resulted dialysate is concentrated 13 times. The obtained dialyzate was considered as stock enzyme for performing all purification steps of *bacillus cereus* MAB5(HQ675025). The protease activity was 4078 u/mg protein/ml. The obtained dialyzate was stored at 4°C.

Dialysis against distilled water

The process of dialysis against water was done to the active dialyzate previously obtained against sucrose crystals (65 ml), then against 0.2 M phosphate buffer pH7.4 until a volume of 280 ml was obtained. Thus, the active enzyme dialyzate was diluted 4.3 times. The specific activity of enzyme was 886.6 unit/mg protein/ml.

Fractional precipitation by ammonium sulphate

The applied six different concentrations of (NH₄)₂SO₄ listed in Table (1) indicated that the most active enzyme protein preparation could be obtained at the (NH₄)₂SO₄ level of 60 %. The obtained precipitate was dissolved into the least amount of water and then subjected to a process of dialysis against distilled water to get rid of the excess of (NH₄)₂SO₄ and then rising up the final volume up to 37 ml in all cases. This step was taken into consideration via the course of purification. Since (NH₄)₂SO₄ fractionation process removed several other enzymes from protein, this increased the specific activity up to 2654.0 unit/mg protein/ml.

Concentration by dialysis against sucrose

The most active (NH₄)₂SO₄ fraction previously obtained

at 60 % saturation was dialyzed against crystals of sucrose until a volume of 2.2 ml was obtained, thus the concentration of the obtained dialysate is 14.9 times. The protease specific activity was determined as 2800 unit /mg protein/ml.

Applying sephadex G-200 column chromatography

The previously prepared dialyzed enzyme preparation was applied to a glass column a previously mentioned. Results of this step are represented in (Table.2) which the most potent peak at fraction number 26 was obtained.

Second purification of peak on sephadex G-100 column chromatography

The active fractions of the peak number 25 obtained after applying the first column of sephadex G-200 were mixed together, concentrated and then applied into a second sephadex G-100 column. Fifty fraction (each of 5 ml) aliquots were eluted and the enzyme assay was performed for each separate fraction. The results (Table 2) showed that only one peak was obtained in which the most active fraction is number 17. However, the most active fractions of this peak were pooled together and then tested for proteolytic activity. The results indicated that the highest specific activity obtained during the purification procedure reached up to **871.2 unit / mg protein/ml**. However, the most active fractions of this peak were pooled together and then tested for protease activity. The results indicated that the highest specific activity obtained during the purification procedure reached up to **565.4 unit / mg protein/ml**.

Characterization of the purified proteases produced by *bacillus cereus* MAB5(HQ675025)

The aim of the some of experiment was carried out to investigate some properties of the purified protease enzymes produced by the most potent strain *bacillus cereus* MAB5(HQ675025). These properties included the effect of dilution on proteolytic activity, substrate concentration, temperature, pH and various heavy metals on proteolytic activity.

Enzyme concentration

The effect of dilution of protease enzyme on the proteolytic activity of the purified protease enzyme was determined. The original stock enzyme preparation was diluted to other seven different descending concentrations (two-fold dilution), then assayed for their activities. The results recorded in **table 3** showed that a continuous increase of enzyme concentration and the maximum activity for protease 697.2 U/ml was obtained at a concentration 0.4000 from original stock preparation.

Effect of substrate concentration

It is obvious from the results recorded in Table (4); the maximum protease activity for protease was obtained at 1% concentration of Chitin 763.8 U/ml. A higher concentration of Chitin (more than 1%) showed a dramatically decrease in protease activity while a lower concentrations e.g. 0.25 and 0.5% (W/V), the activity of protease A attained their maximum activities, beyond which no increase of enzyme activity was recorded.

Effect of temperature

The results (Table 5) showed that the velocity of activity of protease was increased up to 30⁰C which represented the optimum temperature for both enzymes specific activity was 726.1 U/ml for protease. Beyond 30⁰C the activity decreased by the increase of the temperature. However, the protease was showed a responsible activity within the temperature range of 10-50⁰C.

Effect of pH

The results (Table 6) revealed that the optimum pH for activity of protease was attained at pH7.2 with enzyme activity of 597.3 U/ml. Beyond pH7.2 the activity decreased by the increase of the pH. However, the protease was showed a responsible activity within the pH values ranged from 6.8 to 7.6.

Table (1): Ammonium sulfate fractionation pattern of protease by *bacillus cereus*

Fraction No	(NH ₄) ₂ SO ₄ saturation	Mean diameter mm	Enzyme activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg protein/ml)
1	0%	19.3	282.4	1.122	251.6
2	20%	20.0	417.4	0.956	436.6
3	40%	22.6	1041.9	0.962	1083.1
4	60%	26.0	2585.0	0.974	2654.0
5	80%	24.6	2207.5	0.906	2436.5
6	100%	22.3	1126.2	0.890	1265.4

Table (2): Fractionation pattern of protease produced by *bacillus cereus* on sephadex G-100 column chromatogram

Fraction No	Mean diameter (mm)	Enzyme activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg protein/ml)
1-14	NCZ	ND	ND	ND
15	18.3	247.8	0.916	300.0
16	19.0	352.3	0.897	393.8
17	21.0	742.3	0.852	871.2
18	19.6	475.3	0.840	565.4
19	18.0	249.6	0.801	311.0
20	17.0	206.0	0.771	267.0
21	15.6	136.0	0.734	185.0
22-50	NCZ	ND	ND	ND

NCZ = Non clearing zone

ND = Not determined

Table (3): Effect of enzyme concentration on the activity of the purified proteases produced by *bacillus cereus*.

Enzyme concentration (mg protein)	Protease A	
	Mean diameter (mm)	Enzyme activity (U/ml)
0.0030	12.0	59.8
0.0060	13.0	88.5
0.0125	15.0	132.0
0.0250	16.6	176.2
0.0500	17.3	191.8
0.1000	19.6	451.9
0.2000	20.3	447.8
0.4000	21.0	697.2

Table (4): Effect of Chitin concentration on the activity of the purified protease produced by *bacillus cereus*.

Concentration of substrate V/W	Protease	
	Mean diameter (mm)	Enzyme activity (U/ml)
0.25	14.0	76.6
0.50	17.6	244.7
1.00	21.3	763.8
2.00	20.0	457.6
4.00	18.3	302.6
8.00	15.0	95.9

Table (5): Effect of incubation temperature on the activity of the purified proteases produced by *bacillus cereus*.

Temperature ⁰ C	Protease	
	Mean diameter (mm)	Enzyme activity (U/ml)
10	15.0	92.3
20	18.0	235.0
30	21.0	726.1
40	19.0	369.8
50	15.0	92.2

Table (6): Effect of pH on the activity of the two purified proteases produced by bacillus cereus .

pH	Protease	
	Mean diameter (mm)	Enzyme activity (U/ml)
5.2	12.0	38.6
6.0	15.0	95.6
6.4	18.0	242.7
6.8	19.6	482.5
7.2	20.6	597.3
7.6	20.3	476.7
8.0	14.0	74.8

DISCUSSION

A trial was given to obtain the purified protease (s) from the culture filtrate of *B. subtilis* B-6 from one hand and to create an interesting comparative study of the characteristics of the purified enzyme preparations from the other hand. Therefore, *B. subtilis* B-6 strain was subjected separately to a course of protease production, purification as well as investigating the properties of the purified enzymes. The procedure for purification of protease from *B. subtilis* B-6 strain was mentioned previously. It included: cell free filtrate preparation, dialysis, ammonium sulphate fractionation and applying sephadex G-200 column chromatographic technique. The obtained purified protease enzyme(s) were further investigated for both some factors affecting their activities and amino analysis for each purified enzyme preparation. 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 (Kembhawe *et al.*, 1993; Najafi *et al.*, 2005).

Interestingly, the fact that purification steps of protease produced by *B. subtilis* B-6 resulted in having two protease(s) namely peak 25 and peak 31 with specific activities of 727.4 and 460.0 units/ mg/protein/ml. Since purification procedures resulted in having 2 purified enzyme preparations produced from *B. subtilis* B-6. In the present study, there was behavior of the investigated purified protease (Peak 25) especially with the factor of enzyme concentration since the enzyme activity increased simultaneously with the increase of enzyme concentration in the reaction mixture up to certain limit beyond which no increase in enzyme activity could be detected. This behavior is in accordance with the observation of Najafi *et al.* (2005). Upon studying the effect of dilution on the proteolytic activity of the enzymes produced by *B. subtilis*, it was observed that the activity was concentration dependent. There was a significant decrease in the proteolytic activity by the increase of enzyme dilution and this result was in agreement with the reported studies by other investigators (Ammar *et al.*, 1991 and El-Kastawy, 1998). The effect of different incubation temperatures on the proteolytic activity of the crude enzymes produced by *B. subtilis* was studied. The results revealed that the optimum incubation temperatures were 30°C in case of *B. subtilis*, where they showed a significant increase in the proteolytic activity. The obtained results were agreed that reported by El-Kastawy (1998). On the other hand, different optimum incubation temperature were reported by other investigators including 35°C (Gerze *et al.*, 2005), 50°C (Ammar *et al.*, 1991; Ali, 1991), 65°C (Park *et al.*, 1987), 70°C (Sookkheo *et al.*, 2000). The pH value may affect on the proteolytic activity of protease enzymes. In the present study, it was observed that the maximum proteolytic

activity for the crude protease enzymes produced by *B. subtilis* was at the pH range 6.8-7.6 at which there was a significant increase in the proteolytic activity as compared to that at other pH values. However, an optimum proteolytic activity within the pH range 6.8-7.5 was reported by Ghorbel-Frikha *et al.* (2005) and El-Kastawy (1998). Other investigators recorded optimum proteolytic activity at different pH values such as pH 7.8 (Tsujiibo *et al.*, 1990), 8.0 (Ali, 1991), 9.8-10.2 (Kumar *et al.*, 1999), 10.5 (Anwar and Saleemuddin, 1997), and 12-13 (Takami *et al.*, 1989). In the current study, the effect of different gelatin concentration (0.1-4%) on the proteolytic activity of protease enzyme produced by *B. subtilis* revealed that maximum proteolytic activity was obtained in the presence of 1 % gelatin. A higher concentration of gelatin (more than 1%) showed a dramatic decrease in protease activity. The present study was in agreement with studies reported by other investigators (Ammar *et al.*, 1991; Najafi *et al.*, 2005; Soares *et al.*, 2005).

The economic value of this isolate could be established as they could produce high protease yield on cheap media consisting of gelatin as main protein source in addition to other inexpensive ingredients which stimulate high protease production. It is recommended that the study to be extrapolated to include testing the productivity in a bioreactor as well as studying the productivity at a molecular level. Johnvesly *et al.* (2002) used pigeon pea waste as a novel, inexpensive substrate for production of thermostable alkaline protease from *Bacillus* sp.

The protease secreted by a variety of bacterial species and industrially obtainable by fermentation has been used in industrial processing, in analytical chemistry and more recently in chemical medicine, analytical and even medical usage has been applied with the hydrolase (s) enzymes especially in food processing, beverages production and clarification, sewage treatment and many other applications (Najafi *et al.*, 2005). Purification and characterization of the biosynthesized protease from *B. subtilis* B-6 was carried out and their properties were also compared. It is suggested in the light of the previously mentioned applications that the two produced protease could be applied in the fields of medicine and/or industrial purposes. Since this protease has high collagenolytic activity, it can be used for various applications such as: (a) cosmetics: for skin rejuvenation, wrinkle smoothing and dandruff removal, prophylactic on senile skin changes and also removal of the atrophied epithelium from the skin surface, (b) medicine: festering and wet wounds, (c) leather industry: for manufacturing of skin and leather and getting high quality suede, (d) biochemistry: for isolation of cells from many types of animal tissue. This suggestive applicable value will be the subject of further investigations. Further studies were carried out to investigate that factors that

may affect the proteolytic activity of the crude protease enzyme produced by the selected organism.

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