RESEARCH ARTICLE

Characterisation of purified protease from *Bacillus subtilis* BS166

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Abstract: Proteases are enzymes that break up the peptide bonds of proteins to release amino acids. Proteases showing better stability at alkaline pH and higher temperatures have potential applications in several industrial processes. The present study was aimed at determining the kinetic properties of the partially purified protease obtained from *Bacillus subtilis* BS166. *Bacillus subtilis* BS166 can produce protease above 30 °C and at pH 7.0. The protease was extracted with 0.1 M phosphate buffer of pH 7.0 and precipitated with ammonium sulfate. The activity of the purified protease was higher at pH 9.0 and 40 °C and showed zero order kinetics for 10 minutes. When the optimum enzyme concentration was 95.52 µg, a straight line was obtained in the Lineweaver-Burk plot graph. Michaelis constant for the purified enzyme was 50.9 µM and Vmax was 2833.4 pmolmin^{-1} at pH 9.0 and 40 °C.

Keywords: *Bacillus subtilis* BS166, Michaelis constant, protease, Vmax.

INTRODUCTION

Proteases are one of the important groups of industry-valuable enzymes that contribute more in the total enzyme market (Schallmey *et al*., 2004). Proteases (peptidases or proteinases) are enzymes that perform proteolysis. Protein is catabolised by hydrolysis of the peptide bonds that link adjacent amino acids together in the polypeptide chain that forms protein (Paudel & Parajuli, 1999). Proteases control the activity of a variety of proteins, modulate protein-protein interactions, synthesise new molecules, activate cellular information and create molecular signals. Because of these multiple functions, proteases are involved in DNA replication and transcription, cell proliferation, morphogenesis and heat responses of unfolded proteins, angiogenesis, fertilisation, ovulation, healing of wounds, stem cell mobilisation, blood coagulation, inflammation, haemostasis, immunity, necrosis, senescence and defence. Consistent with these important contributions of proteases in cells and survival and death of all organisms, alterations in proteolytic enzyme systems underlie multiple pathological diseases (Christeller, 2005). Many proteases are used as potential drug targets and as biological markers. Proteases are widely distributed in plants, animals and microbes. In higher organisms, almost 2 % of the genes code for proteases. The proteases are useful in various ways and their applications in life show an increasing trend. Proteases are used in degumming of silk goods, manufacturing liquid glue, preparation of cheese, medicines and cosmetics, tenderisation of meat and in agriculture as growth promoters. The major sources of these proteases are microbes.

Bacillus species produce a huge variety of extracellular enzymes such as protease, amylase and xylanase, which are of great industrial importance. Bacterial enzymes are thermostable than fungal proteases. Members of the genus *Bacillus* are heterogeneous and are versatile in their adaptability to the environment. There are a lot of factors that influence metabolic processes and enzyme synthesis and function. There is a huge expectation on the thermophilic and extremely thermophilic microorganisms and their enzymes (Grubb & Lofberg, 1982). *Bacillus subtilis* BS166 is a Gram-positive, rod shaped, endospore-forming bacteria. It is catalase positive and commonly found in soil including low nutrient soil. Unlike several other well-known species, *Bacillus subtilis* BS166 has been classified as an obligate aerobe. Due to its association with soil particles, it could be transferred to plants, food, animals and even marine and freshwater habitats (Barrow & Feltham, 1993; Madigan & Martinko, 2005).

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Proteases from diverse *Bacillus* species have been extracted, purified and characterised and the substrate specificity, thermal stability, pH stability, downstream processing procedures and low cost have been investigated (Hadder *et al*., 2009). Such properties increase the utility of proteases for industrial applications. Proteases showing better stability at alkaline pH and higher temperatures have potential applications in several industrial processes (Schallmey *et al*., 2004). For large scale production of proteases in the industrial practice, scientists are looking for microbes that can produce alkaline and thermostable protease enzymes at a wide range of pH values. These microbes can also be used in biotechnological applications. Theromostability and alkalinic nature of the protease enzyme produced by *Bacillus subtilis* BS166 strain is not known and these properties need to be investigated. Therefore the objective of this research was to purify protease from *Bacillus subtilis* BS166 strain by ammonium sulfate precipitation and to study the kinetic properties of the enzyme.

METHODOLOGY

Source of *Bacillus subtilis*

The culture of *Bacillus subtilis* BS166 strain was provided by Dr Sandeepa Kanitkar, Department of Biological Science, University of British Columbia, Canada.

Initial screening of growth and potential protease production by *Bacillus subtilis* BS166 strain

The pure culture of *Bacillus subtilis* BS166 strain was screened for growth and the production of extracellular protease using skim milk agar plates. The pure cultures were streaked as a line on the skim milk agar plates and the plates were incubated at 37 °C for 24 hrs.

Protease production by *Bacillus subtilis* BS166

Ten milliliters of an overnight culture of *Bacillus subtilis* BS166 was used to inoculate a 0.5 L Erlenmyer flask containing 490 mL aliquots of liquid production medium, which consist of glucose 0.5 % (wt/vol), peptone 0.75 % (wt/vol), KH_2PO_4 0.5 % (wt/vol), $MgSO_4.7H_2O$ 0.05 % (wt/vol) and $FeSO_4.5H_2O$ 0.01 % (wt/vol) at pH 7.0 and maintained at 37 °C for 24 hrs in a shaker incubator. The pH of the medium was adjusted by 1 N NaOH or 1 N HCl. After the completion of fermentation, the whole fermentation broth was centrifuged at 15,000 rpm at 4 °C for 15 min and the clear supernatant was recovered. The supernatant was used as the crude enzyme for further studies.

Analytical methods - Bradford assay of crude extract

A working solution of bovine serum albumin (BSA) was made by diluting the standard BSA solution of 1 mg/mL to 0.1 mg/mL. In 5 test tubes 200 µL, 400 µL, 600 µL, 800 µL and 1 mL of the BSA working solution was taken and named 1-5. The volume was maintained at 1 mL by adding distilled water. A blank was set with only distilled water. 500 µL of the crude extract was taken for Bradford assay and 500 µL of sterile distilled water was added to make the final volume of 1 mL. Then 5 mL of the Bradford reagent was added and after 5 min of incubation, the absorbance was measured with the spectrophotometer at 595 nm. 50 µL of BSA (1 mg/mL) standard solution and around 20 µL of crude extract was taken to calculate the proteolytic activity of the crude enzyme. The BSA and enzyme were mixed and the volume was maintained at 200 µL by the addition of phosphate buffer. The mixture was incubated for 30 min and 2.3 mL of the Bradford reagent was added. After 5 min, the absorbance was measured with the spectrophotometer at 595 nm against blank with reference to two controls: enzyme only and BSA only. The activity of protease was calculated with the following equation (Bradford, 1976). One unit of protease activity is defined as the amount of enzyme that produces 1 µmol of tyrosine per minute at pH 9.0 and 60 °C.

$$\text{Protease activity} = \frac{\text{Amount of BSA chopped (µg)} \times \text{Volume of enzyme (mL)}}{\text{Incubation time (min)}}$$

Precipitation by ammonium sulfate and dialysis

The culture supernatant was taken in a conical flask. Solid ammonium sulfate was gradually added to the conical flask with stirring using a sterile magnetic stirrer, to make up to 70 % saturation. Then it was kept in the refrigerator for complete precipitation. The protein precipitate obtained was separated by centrifugation at 15,000 rpm for 15 min at 4 °C. The supernatant was discarded and the pellet obtained was dissolved in 15 mL of 0.1 M phosphate buffer and dialysed overnight against 0.1 M phosphate buffer of pH 7.0. Salt containment of the solution was removed by overnight dialysis in 0.1 M phosphate buffer. The protease activity was determined by using standard Bradford assay method (Bradford, 1976).

Characterisation of partially purified protease

Effect of pH

The effect of pH on protease activity was measured in buffers at different pH values [Citrate-phosphate buffer (pH 3.0 to 6.0), sodium phosphate buffer (pH 7.0), Tris buffer (pH, 8.0 and 9.0), carbonate-bicarbonate buffer (pH 10.0 – 12.0)] and incubated at optimised conditions. Thirty microliters of the enzyme extract was mixed with 50 µL of BSA standard solution (1 mg/mL) and the volume was maintained at 200 µL by adding different buffers of different pH. After incubation for 30 min, 2.3 mL of Bradford reagent was added and the protease activity was determined by using standard Bradford assay method (Bradford, 1976).

Effect of temperature

The effect of temperature on the protease activity of 70 % ammonium sulfate precipitated extracts was characterised at different temperatures. For different temperatures the refrigerator (4 ºC), room temperature (20 ºC), incubator (40 ºC) and oven (60 ºC, 80 ºC, 100 ºC) were used. Thirty microliters of enzyme extract was mixed with 50 µL of BSA standard solution and the volume was maintained at 200 µL by adding phosphate buffer and the protease activity was determined by using standard Bradford assay method (Bradford, 1976).

Effect of enzyme concentration

Different concentrations of the protease enzyme (10, 20, 40, 60, 80, 100 and 120 gL^{-1}) were prepared with phosphate buffer (pH 7.0, 0.1 M) at optimum pH and they were mixed with 50 µg of BSA standard solution to react at the optimum temperature for optimised time. The protease enzyme activity was determined by using standard Bradford assay method (Bradford, 1976).

Effect of substrate concentration

Different concentrations of the BSA solution was prepared (20, 40, 60, 80, and 100 µgL^{-1}) with phosphate buffer

(pH 7.0, 0.1 M) at optimum pH and they were allowed to react with the diluted enzyme at the optimum temperature for optimised time. The protease enzyme activity was determined by using standard Bradford assay method (Bradford, 1976). The Michelis constant and Vmax for the purified protease were calculated.

RESULTS

Confirmation of the *Bacillus subtilis* BS166 strain utilising protein

The pure culture of *Bacillus subtilis* BS166 strain produced clear zones in the skim milk agar plates after 24 hours.

Ammonium sulfate salt precipitation of crude extract

The protease enzyme activity of the crude enzyme was 69.6 pmolmin^{-1}mL^{-1}. To the crude protease, solid $(NH_4)_2SO_4$ was added. Precipitation of the protein increased but the specific activity increased with $(NH_4)_2SO_4$ saturation (Table 2). The protease activity of 70 % ammonium sulfate precipitated extract was 432.5 pmolmin^{-1}mL^{-1} (Table 1). The crude enzyme sample precipitated with 70 % $(NH_4)_2SO_4$ saturation showed the highest specific activity (305.87 pmolmin^{-1}mL^{-1}) (Table 2).

Table 1: Protease activities of crude and ammonium sulphate precipitated extract

	Remained BSA in µg	Chopped BSA in µg	Activity in pmolmin^{-1}mL^{-1}
Crude	44.2	5.3	69.6
After ammonium sulphate purification	22.4	26.6	432.5

Table 2:   Summary of protein and protease purification using ammonium sulphate

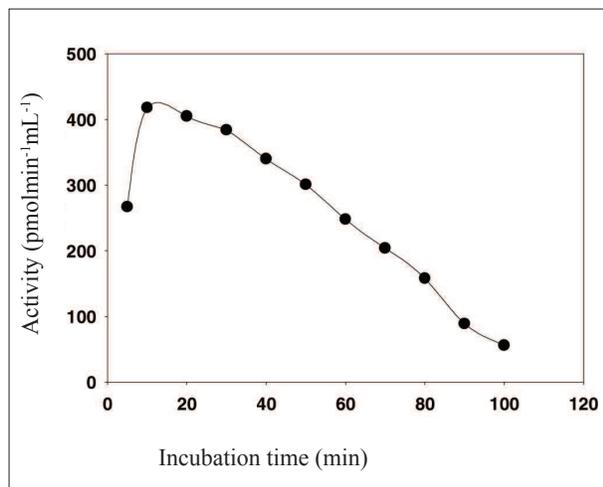|	Volume (mL)	Protein concentration (mg/mL)	Total protein (mg)	Activity (pmolmin^{-1} mL^{-1})	Total activity (pmolmin^{-1} mL^{-1})	Specific activity (pmolmin^{-1}mL^{-1})	Purification factor	Overall yield (%)
Crude	420	0.302	126.84	69.6	29232	230.46	1	100
Purified	20	1.414	28.28	432.5	8650	305.87	1.33	29.6

Figure 1: Effect of time of incubation on protease activity of 70 % ammonium sulfate precipitated extract
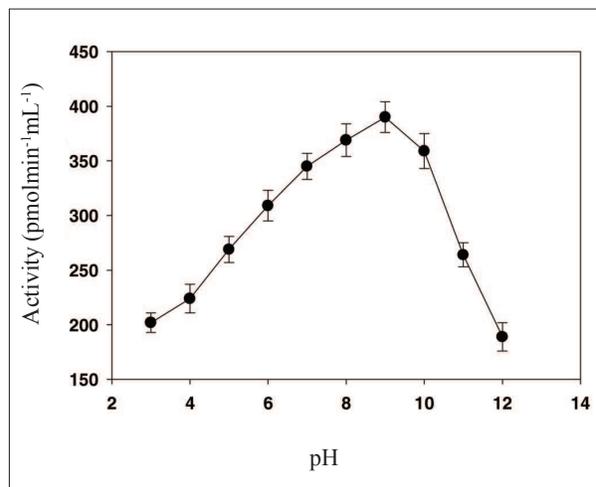

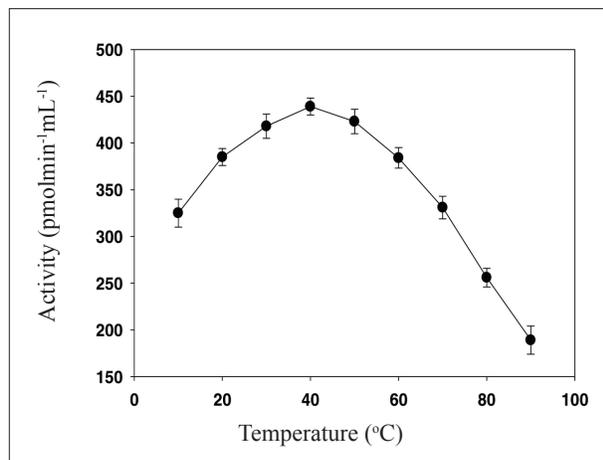**Figure 2:** Effect of pH on protease activity of purified enzyme

Figure 3: Effect of temperature on the purified protease activity
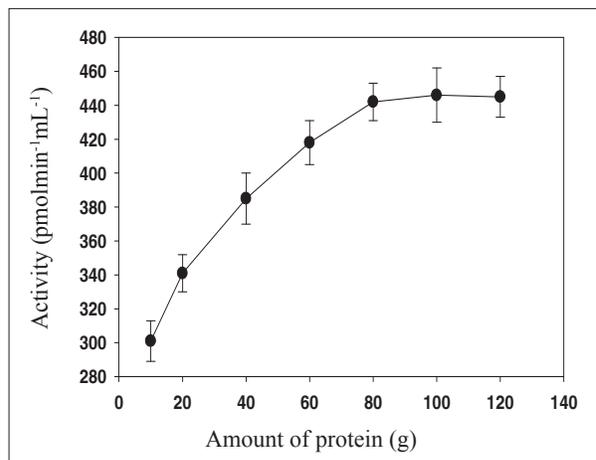

Figure 4: Effect of enzyme concentration on the purified protease activity

Effect of time

Purified protease showed zero order kinetics for 10 minutes (Figure 1). Therefore the incubation time for the kinetic studies was fixed as 5 minutes in the following experiments.

Effect of pH

When the pH varied from 3.0 to 12.0, the activity of protease increased up to pH 9.0 and further increase of the pH decreased the enzyme activity. Thus for further studies a pH of 9.0 was selected for purified protease. Protease enzyme showed activity between pH 3.0 to 10.0. However the enzyme showed 75 % of its original activity at pH values

between 7.0 and 10.0 (Figure 2). Since the highest protease activity was obtained at pH 9.0, it was selected for further studies.

Effect of temperature

The protease enzyme activity was assayed at different temperatures ranging from 40 – 90 °C at pH 9.0. The activity of protease increased up to 40 °C and further increase in temperature decreased the enzyme activity. Purified protease exhibited a temperature profile with a sharp peak of maximal activity at 40 °C (442.98 pmolmin^{-1}mL^{-1}) and showed activity between 40 – 90 °C (Figure 3).

Effect of enzyme concentration

The protease enzyme activity was assayed at different concentrations of the protein ranging from 10 – 120 gL^{-1} at pH 9.0 and a temperature of 40 $^{\circ}$C. The activity of protease increased up to 80 gL^{-1} and further increase in the protein concentration decreased the enzyme activity (Figure 4).

Effect of substrate concentration

When the substrate concentration was increased from 20 – 100 μgL^{-1} at pH 9.0, the activity of protease increased up to 40 μgL^{-1} and reached the maximum velocity at 60 μgL^{-1} (Figure 5). Michaelis constant for the purified enzyme to soluble protein was 50.9 μM and Vmax was 2833.4 $pmolmin^{-1}$ at pH 9.0 and 40 $^{\circ}$C. Therefore 40 gL^{-1} substrate concentration was used for the assay for further studies of enzyme activity.
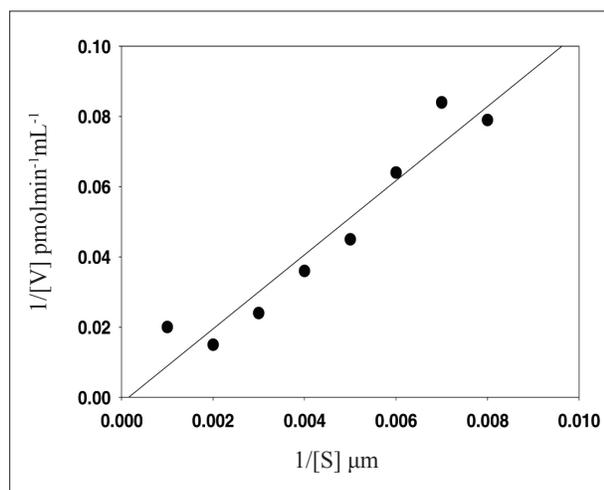

Figure 5: Lineweaver-Burk plot of the purified protease activity at pH 9.0 and 40 $^{\circ}$C using different concentrations of substrate

DISCUSSION

The pure culture of *Bacillus subtilis* BS166 strain produced clear zones in the skim milk agar plates after one day of incubation and this confirms the capacity of the strain to grow and utilise protein efficiently. Many types of salts have been employed for protein separation and purification through salting-out, and different proteins precipitate at different salt concentrations. Since $(NH_4)_2SO_4$ is a cost effective salt that is widely used for purification of proteases from bacteria, it was used in this study (Schallmey *et al*., 2004; Kapilan & Arasaratnam, 2014). Since 70 % of ammonium sulfate saturation generally gives a higher yield than the other saturations, it was decided to use this saturation in the experiment (Kapilan, 2015). The specific activity of 70 % ammonium sulfate precipitated fraction was significantly higher as compared to that of crude extract indicating purification of protease by 1.33 fold. The optimal pH of the protease was 9.0 and this suggests that the purified protease could be classified as an alkaline protease. The optimal temperature for protease activity was 40 $^{\circ}$C. The protease remained active even at 90 $^{\circ}$C. Therefore it could be recommended that the purified protease is slightly thermophilic. The stability of the enzyme at higher temperatures might be due to the presence of thermotolerant residues in the purified extract. Sometimes ammonium sulfate might stimulate the formation of these residues. Some studies have reported the presence of higher amount of cysteine residues in some bacterial proteases (Murray *et al*., 1999). The initial increase in protease activity with temperature might be due to the collision between the enzyme and substrate. The increase in temperature causes increase in collision between enzymes and substrates (Mehrato *et al*., 1999). Beyond these optimal temperatures the activities were decreased as a result of denaturation of proteases due to heat (Karki *et al*., 2009).

An alkaline protease from *B. subtilis* was observed to be stable in the pH range of 8.0 – 11.0 with the highest activity at pH 10.0 (Adinarayana *et al*., 2003). An extracellular alkaline protease from a thermophilic *Bacillus* strain has been purified using 80 % ammonium sulfate precipitation followed by CM-cellulose and DEAE-cellulose ion exchange column chromatography that yielded 16.5 fold purification with the specific activity ranging from 417 to 6900 U/mg (Kamran *et al*., 2014; Hashem *et al*., 2015). The enzyme activity gradually reduces indicating inactivation of enzyme with time. A slightly linear line was obtained for the effect of enzyme concentration on protease activity indicating that the enzyme concentration enhances the rate of reaction. The linear line obtained for the effect of substrate concentration on protease activity indicated that the rate of reaction increases with the increase in substrate concentration. The low Michaelis constant (Km) value of proteases indicated that the substrate is tightly bound to the enzyme.

CONCLUSION

The protease enzyme produced by pure *Bacillus subtilis* BS166 strain was purified by 70 % of ammonium sulfate salt with a purification fold of 1.33. The optimum activity of the purified protease was observed at pH 9.0 and

temperature 40 °C. The purified protease showed zero order kinetics for 10 minutes and was active even at the temperature of 90 °C. Michaelis constant of the purified enzyme to soluble protein was 50.9 μM and Vmax was 2833.4 pmolmin^{-1} at pH 9.0 and 40 °C.

Acknowledgement

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