African Journal of Biotechnology Vol. 5 (24), pp. 2433-2438, 18 December 2006
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2006 Academic Journals

Full Length Research Paper

Purification and characterization of a protease from *Thermophilic bacillus* strain HS08

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Accepted 19 October, 2006

The purification and characterization of a thermophilic neutral protease from *Thermophilic bacillus* strain HS08, originally isolated from a soil sample collected from the Tulufan Crater of China, is presented in this paper. The purification steps included ammonium sulfate precipitation, with columns of DEAE-Sepharose anion exchange chromatography and Sephacryl S-100HR on AKTA purifier 100 protein liquid chromatography. The method gave a 4.25 fold increase of the specific activity and had a yield of 5.1%. The molecular weight of the protease was found to be around 30.9 kDa by SDS-PAGE technique. The optimal pH and optimal temperature of the protease were at pH 7.5 and 65°C, respectively. The protease was found stable during the 1 h incubation at 50°C. The protease activity showed wide range of variation in the presence of different reagents: it was inhibited remarkably by EDTA or PMSF and was almost activated by 2 mM Zn^{2+}, even though it was only marginally inhibited by other inhibitors. We concluded that the protease was a Zn^{2+}-acitived serine protease. Substrates specificity tests indicated that azocasein was the best substrate among the three substrates tested (azocasein, casein, and BSA).

Key words: Neutral protease, purification, characterization, ***Thermophilic bacillus***, thermophilic protease, serine protease.

INTRODUCTION

Proteases (EC 3.4) are enzymes that hydrolyze proteins to short peptides or free amino acids. The very importance of enzyme as sources for industrial applications has been well recognized and it was reported that proteases count for nearly 65% of the world enzyme market (Rao et al., 1998). Proteases have a wide range of applications, particularly in food, detergent, weave, leather, pharmaceutical and chemical industries. For example, proteases are widely applied to increase the tenderness of meat. Commercial proteases are mostly produced from various bacteria and it was reported that about 35% of the total microbial enzymes used in detergent industry are the proteases from bacteria sources (Ferrero et al., 1996). Proteases are commonly classified according to their optimum pH: acidic protease, neutral protease and alkaline protease. There have been extensive researches on the studies of properties and functionalities of acidic or alkaline proteases. One particular interest is the production of alkaline protease from bacillus for applications in detergent industry (Ferrero et al., 1996; Manachini and Fortina, 1998; Johnvesly et al., 2002; Fu et al., 2003). Thermophiles such as ***Bacillus stearothermophilus*** (Boonyanas et al., 2000), ***Thermus aquaticus*** (Gabriela et al., 2003), ***Bacillus licheniformis*** (Ferrero et al., 1996), ***Bacillus pumilus*** (Kumar, 2002), and ***Thermoanaerobacter yonseiensis*** (Hyenung et al., 2002) have been studied for their capability in producing thermostable proteases. Biochemical properties of the enzymes produced from these thermophilies have also been well investigated. Because of their high activity and stability at elevated temperatures, the thermophilic proteases can also be

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used as ideal models for studying thermal stability of protein (Rao et al., 1998). Among these enzymes, the neutral proteases have been extensively studied not only for industrial production such as detergent, weave processing, meat tendering and reducing the risk of contamination by other organisms at high temperature, but also for the elucidation of mechanisms involved in thermostability of enzymes (Helmann, 1995; Imanaka et al., 1986).

In this paper, we report our recent progress on the purification and biochemical characterization of a novel protease from ***Thermophilic Bacillus*** strain HS08.

MATERIALS AND METHODS

Bacterial strain and enzyme production

T. bacillus strain HS08 was first isolated in this laboratory from a soil sample collected from Toulufan Crater, Xingjiang Province, China.(Huo et al., 2003). The strain was then left to grow in a medium containing 0.5% (w/v) yeast extract and 1.0% (w/v) peptone. The growing medium mixture was composed of 0.5 g/l glucose, 0.4 g/l Na_2HPO_4, 0.085 g/l Na_2CO_3, 0.0 2g/l $ZnSO_4$, 0.0 2g/l $MgSO_4$, 0.02 g/l $CaCl_2$ and has a pH value of 7.2. The culture was incubated in aerated vessels at 46°C for 16 h under a continuous stir (at 200 rpm) and then centrifuged at 14,000 g for 30 min at a cool temperature of 4°C (Hitach CF15R, Hitachi Koki Co.Ltd., Japan). The supernatant was assayed for proteolytic activity and used for further purification.

Purification of thermostable proteases

All purification works were carried out at 4°C. The supernatant was precipitated in ammonium sulphate solution ($(NH_4)_2SO_4$) of 80% saturation. The precipitates were collected after centrifugation (14,000 g, 30 min, 4°C) and re-dissolved in 10 ml 50 mM pH 7.2 Tris-HCl buffer. The solution was dialysed in the same buffer for 12 h at 4°C before applying on a DEAE-Sepharose Fast Flow column (1.6×40 cm) at AKTA purifier 100 protein liquid chromatograph (Amersham Biosciences AB, Sweden). The protease was eluted using a 50 mM pH 8.5 Tris-HCl buffer at a rate of 0.3 ml/min and in a linear gradient of 0 ~ 1 M NaCl. The fractions containing protease were pooled and precipitated again with 80% saturation of ammonium sulphate solution. The mixture was left overnight at 4°C and then centrifuged at the same conditions as stated earlier. The collected precipitates were dissolved in 10 ml 50 mM pH 7.2 Tris-HCl buffer. The obtained solutiojn was applied on a Sephacryl S-100HR (1.6×40 cm) and eluted with 50 mM pH 7.2 Tris-HCl buffer at a rate of 0.4 ml/min. The protein content was determined using the method previously described (Shakir et al., 1994), with bovine serum albumin (BSA) as the standard.

Protease assay

A mixture of 400 µl casein solution (2% w/v in 50 mM Tris-HCl buffer pH7.2) and 100 µl of the sample were added to a tube. The reactions were carried out at 65°C for 10 min and then terminated by the addition of 1 ml 10% trichloroacetic acid (TCA, w/v). The mixture was centrifugation at 14,000 g for 20 min and a total of 200 µl supernatant was carefully removed to measure tyrosin content using a Folin-phenol method (Michel et al., 1986). One unit of protease activity (U) was defined as the amount of enzyme that hydrolyzed casein to produce 1 µg tyrosin within 1 min at 65°C.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 12% (w/v) isolation gel and 5% (w/v) concentration gel according to the method of Laemmli where 2-mercaptoethanol was used as reducing agent (Grebeshova et al., 1999). The molecular weight was determined by interpolation from a linear semi-logarithmic plot of relative molecular mass versus the R_f value (relative mobility) on Minibis Bioimaging System (DNR Bio-Imaging Systems Ltd., Israel) and the following proteins were used as standards: phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (20.1 kDa), and β-lactalbumin (14.4 kDa).

Effect of temperature and pH on protease activity

In order to determine the optimum temperature, protease activity was measured in 50 mM pH 7.2 Tris-HCl buffer solutions at 30, 40, 50, 55, 60, 65, 70, 75 and 80°C, respectively. Protease activity was also measured at 65°C in the following buffer systems: 50 mM sodium acetate (pH 4.0~5.5), 50 mM sodium phosphate (pH 6.0 ~ 7.0) and 50 mM Tris–HCl (pH 7.5 ~ 9.0) to measure the optimum pH. To test the thermostability of the protease, the purified protease was incubated at various temperatures ranging from 40 to 80°C for 10 to 60 min, then cooled in ice-water and the residue activity was measured under standard assay conditions.

Substrate specificity

The hydrolysis activity toward a variety of proteins including 2% (w/v) BSA, 2% (w/v) casein and 2% (w/v) azocasein by the purified protease was assayed under standard assay conditions.

Determination of protease type

To determine the type of protease that yielded the proteolytic activity, the purified protease was pre-treated for 30 min at 37°C with the following reagents respectively before assay were run: 2 mM PMSF, 2 mM NBS, 2 mM phenylgloxal hydrate, 2 mM WRK, 2 mM TNBS, 2 mM $CaCl_2$, 2 mM $CuSO_4$, 2 mM $ZnSO_4$, 2 mM Cys, 2 mM Na_2.EDTA and 2 mM 2-mercaptoethanol. The remaining activity was measured under standard assay conditions.

RESULTS AND DISCUSSION

Purification of the protease

The supernatant from the cell culture was precipitated with 80% saturation of $(NH_4)_2SO_4$, then the precipitates were dissolved and dialyzed in 50 mM Tris-HCl buffer pH 7.2. There were three protein peaks and three protease activity peaks (Figure 1) when the dialyzed protease was applied on a DEAE-Sepharose Fast Flow column. The maximum protease activity peak (peak I) was eluted at 0.3 M NaCl, the other two relative weak protease peaks (peak II and peak III) were eluted at 0.6 and 0.8 M NaCl.

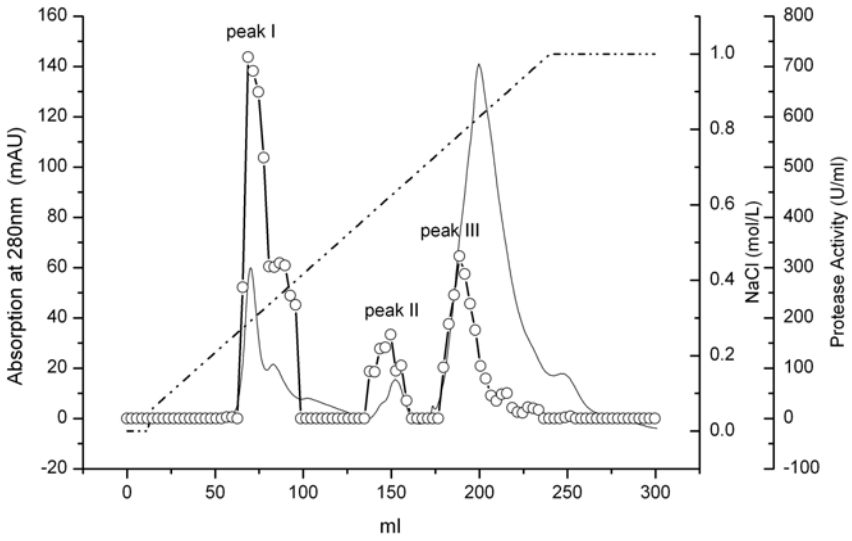


Figure 1. Chromatograph of the thermophilic protease by 1.6×40 cm column with DEAE-Sepharose Fast Flow at 0.3 ml/minute. Protease activity (O); NaCl gradient (---); protein concentration with UV_{280} (–).

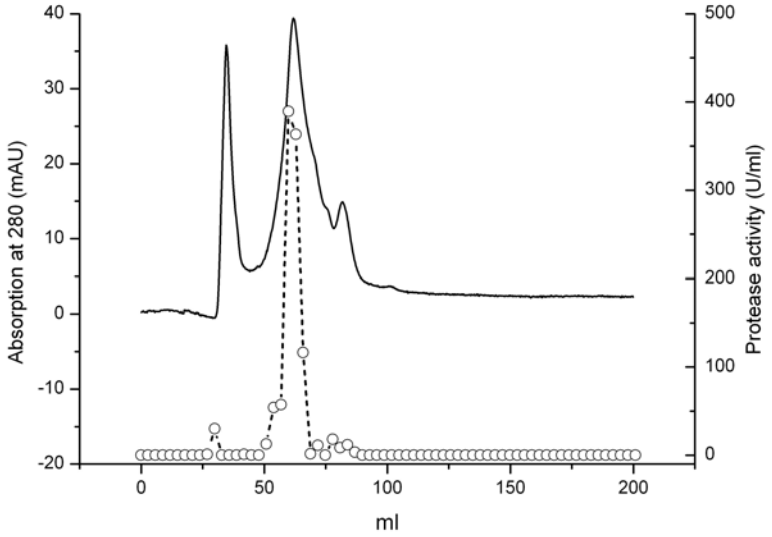


Figure 2. Chromatograph of protease by 1.6×40 cm column with Sephcryl S-100HR at 0.3 ml/minute. Protease activity (O); protein concentration with UV_{280} (–).

The fractions of the peak I on the DEAE-Sepharose column was collected and precipitated with 80% saturation of $(NH_4)_2SO_4$, dissolved and dialyzed in 50 mM Tris-HCl buffer. The solution was applied on a Sephacryl S-100 HR column. Only one strong protease activity peak was detected, although there appeared three protein peaks (Figure 2). The protease activity peak fractions was collected and run in a SDS-PAGE gel. A single band was obtained with a molecular mass of approximately 30.9 kDa (Figure 3). And the protease was purified to 4.25-fold with a 5.1% yields (Table 1).

Effect of temperature and pH on protease activity

The effect of temperature on the activity of purified protease was analyzed at 30 to 80°C (Figure 4). The results showed a bell-shaped temperature dependence with an optimal activity at 65°C. When the reaction temperature reached higher than 75°C, there was a sharp decrease of its activity and we observed only 30% of the maximum activity at 80°C. The protease had a relatively broad temperature adaptability ranging from 55 to 75°C. The heat stability of the protease at 40 to 80°C

Table 1. Purification of neutral protease from ***T. bacillus*** strain HS08.

Purification steps	Volume (ml)	Protein (mg/ml)	Enzyme activity (U/ml)	Total Enzyme activity (U)	Specific activity (U/mg)	Purification folds	Yield (%)
Crude extraction	50	3.84	11892	594600	3097	_	100
80% ammonium sulfate fraction	14	2.60	14390	201460	5535	1.79	33.9
DEAE-Sepharose Fast Flow	32	0.24	2360	75520	9833	3.18	12.7
Sephacryl S-100HR	40	0.058	764	30560	13172	4.25	5.1

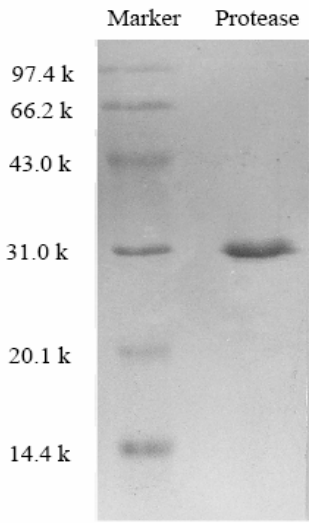


Figure 3. SDS PAGE chromatograph of the thermophilic neutral protese. Marker: standard molecular protein; Protease: active fractions eluted from the Sephcryl S-100HR column.

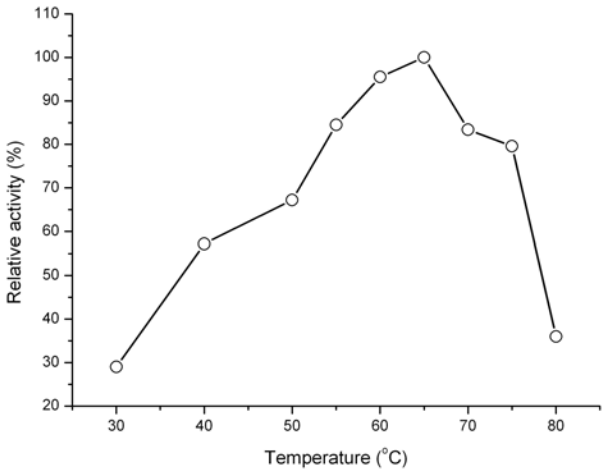
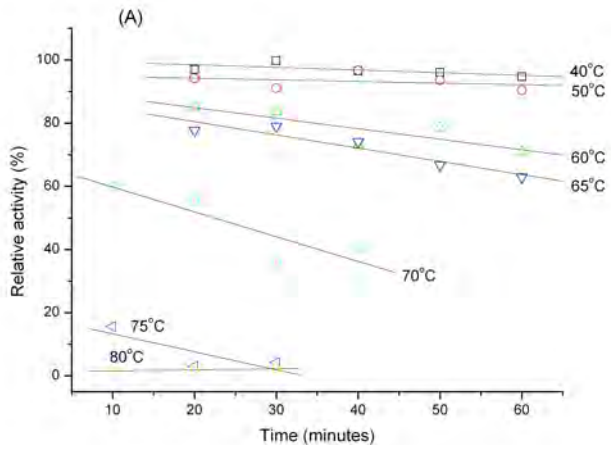


Figure 4. Effects of temperature on the protease activity.



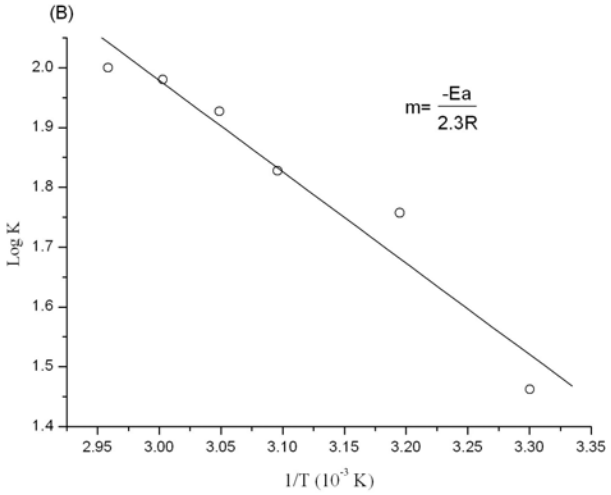


Figure 5. Effects of temperature on the stability of protease activities.

was shown in (Figure 5A). The results demonstrated that it had good stability between 40 and 65°C. After 60 min incubating at 65°C it still had about 75% activity. So the protease can be classified as thermophilic. The temperature-dependence curve of the protease was further examined using the Arrenhenius equation between 30 and 70°C (Figure 5B). The value of the activation energy (Ea) for the catalytic step was calculated to be 29.17 kJ mol^{-1} from the slope of an Arrhenius plot of log ***V***max versus reciprocal temperature in the temperature range from 30

Table 2. Effect of some reagents on the protease activity.

Reagents	Concentration (mM)	Relative activity (%)	Reagents	Concentration (mM)	Relative activity (%)
Blank		100	PMSF	2	4
			NBS	2	85
$CaCl_2$	2	87	WRK	2	94
$CuSO_4$	2	83	Phenyloxal hydrate	2	87
$ZnSO_4$	2	109	DTT	2	100
Cys	2	81	TNBS	2	94
Na_2. EDTA	2	6			
2-mercaptoethanol	2	86			
SDS	2	84			

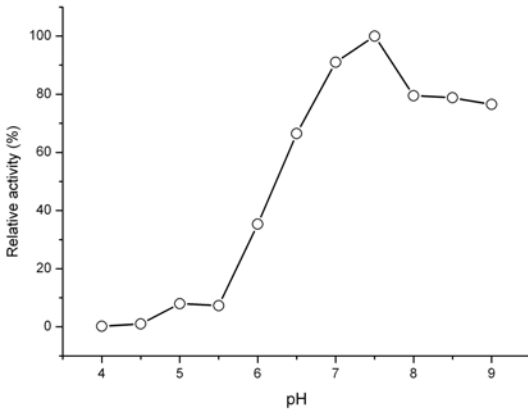


Figure 6. Effects of pH on the protease activity.

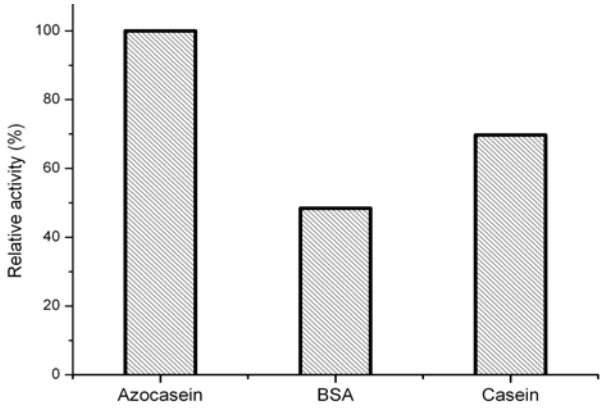


Figure 7. Protease activities on various protein substrates.

to 70°C. Studies of the influence of pH on the enzyme activity have also been carried out and the results are shown in Figure 6. We can see that the activity of the protease was very low at pH values between 4.0 and 5.5, and then increased sharply beyond pH 5.5. The protease remained active between pH 7 and 9 but had its maximum activity at pH 7.5.

Substrate specificity

The ability to hydrolyze several protein substrates is an important criterion of protease potency (Grebeshova, 1999). Figure 7 compares the digestive capability of this protease on three proteins. It is evident that the protease had better digestive ability on azocasein than that on casein or BSA. Under the same assay condition, the relative activity of the protease only reached 70% (for casein substrate) and 50% (for BSA substrate) of that when azocasein was used as the substrate.

Determination of protease type

In order to determine the nature of the protease, activities were also measured in the presence of different reagents. It was found that 2 mM Na_2·EDTA and 2 mM PMSF only gave a relative activity of 43 and 6 %, respectively (Table 2). However, both 2 mM Zn^{2+} showed enhanced enzyme activity, where the relative activity was recorded to be 109%. Other reagents such as Ca^{2+}, Cu^{2+}, NBS, WRK, TNBS and 2-mercaptoethanol did not show significant inhibitory effects against the protease. It is, therefore, reasonable to conclude that the protease of this study was a Zn^{2+}-activated serine protease.

Conclusion

A thermophilic neutral protease from ***T. bacillus strain*** HS08 was purified with DEAE-Sepharose anion exchange chromatography and Sephacryl S-100 HR liquid chromatography. The molecular weight was found to be around 30.9 kDa by SDS-PAGE technique. The optimal pH and optimal temperature of the protease were at pH 7.5 and 65^{o}C, respectively. The protease was found stable during the 1 h incubation at 50°C. The protease activity was inhibited by EDTA or PMSF and was almost activated by 2 mM Zn^{2+}, even though it was only marginally inhibited by other inhibitors. We concluded that the

protease was a Zn^{2+}-activiated serine protease. Substrates specificity tests indicated that azocasein was the best substrate among the three substrates tested (azocasein, casein, and BSA).

ACKNOWLEDGMENT

This work was supported by Zhejiang Provincial National Science Foundation of China. The project number was Y405083.

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