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Purification and Characterization of an Extracellular Protease from *Bacillus pumilus* CN8

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ABSTRACT - The protease produced by a *Bacillus pumilus* CN8 strain was purified by DEAE-Cellulose-52 ion exchange. It has a molecular weight of approximately 96,920 Dalton. In the present study, this protease showed strong activity over a broad range of pH (6.5-9.5) and temperature from 40°C to 60°C, and the protease performed the maximal activity at pH 7.3 at 42°C. The effect of metal ions on protease activity showed that K^+ could slightly increase the protease activity, and other ions such as Zn^{2+}, Fe^{2+}, Na^+, Ca^{2+}, Mg^{2+} had no significant activation or inhibition to the protease ($P > 0.05$), and the more important is that Cu^{2+}, Mn^{2+}, Sn^{2+}, Cd^{2+} had a strong inhibitory effect on the protease activity.

Key words: *Bacillus pumilus*, Protease, Purification, Characterization

Proteases, a large group of enzymes in all organisms, are one of the most important groups of industrial enzymes[1]. These enzymes play important role in blood-clotting and metabolic control, and also can either break specific peptide bonds, depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids. Proteases are widely applied in various fields of industry, such as detergent formulations, leather preparation, protein recovery or solubilization, food processing industries and organic synthesis and so on[2-5].

Microbial proteases, secreted by bacteria to break the proteins down into their constituent monomers, make approximately 40% of the total worldwide production of enzymes[6]. Most of the microbial proteases from *Bacillus* species are the most widely exploited industrial enzymes with major applications in detergents[7]. All these enzymes exhibit stability in the presence of various components of detergents and are active at different conditions. Since the first alkaline protease Carlsberg extracted from *Bacillus licheniformis* was commercialized as an additive in detergents in the 1960s, more alkaline proteases have been purified and characterized, also significant biological activity, stability, broad substrate specificity, short period of fermentation, and lower-stream application technology have been simultaneously demonstrated[8,9].

It is essential to use protease to degrade porcine hemoglobin in industry in China today. As a major export of the pork commodity, the livestock product industry makes great profits while also bringing significant environmental pollution. Numerous previous studies have been reported that various strains of *Bacillus cereus* could produce enzymes with properties which suggest that they are sensitive to metal chelating agents[10,11]. After years of hard work for selection and breeding of our research team, the strains of *Bacillus pumilus* CN8 were obtained from contaminated farm soil by pig blood for the porcine blood hemoglobin degradation. The protease isolated from culture filtrates of *Bacillus pumilus* CN8 was an extracellular enzymes[12]. The performance of proteases depends on number of factors including the pH, temperature, metal ions, molecular structure and weight of the proteases, and chemical denaturants[13,14]. The purpose of this study was to determine the purification and characterization of an extracellular protease from *Bacillus pumilus* CN8.

Materials and Methods

Strain and growth conditions

Bacillus pumilus CN8, isolated by Huazhong Agricultural University, Wuhan, China, was used as a protease producer. The optimal fermentation medium for this strain contains 1% glucose, 0.04% K_2HPO_4, 0.05% NaCl, 0.06% KH_2PO_4, 0.01% $MgSO_4$, 0.002% $FeSO_4$, 0.1% Span20, 1% hemoglobin with a pH value of 6.5. The strain was cultured in a 1 liter fermenter

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and incubated at 37°C for 72h.

Materials

DEAE-Cellulose-52 used in this study was manufactured by Whatman (Maidstone, UK). Molecular marker was obtained from Pharmacia Co. Ltd (Uppsala, Sweden). Other reagents were purchased from Shanghai Watson Biotechnology Co. Ltd (Shanghai, China).

Protein assay

Protein concentration was determined by the Bradford assay with bovine serum albumin as a standard[15].

Protease assay

The protease activity was assayed on the basis of the amount of digested casein. The reaction was started by adding 1 ml enzyme which was contained in a solution of phosphate buffer (pH 7.0) to 1 ml of casein diluted at 4 mg/ml concentration in the same buffer. After incubation for 10 min at 40°C, 2 ml CCl_3COOH was added to terminate the reaction. Then, 5 ml of Na_2CO_3 diluted at 0.4 mol/l concentration and 1 ml of Folin-hydroxybenzene were added after filtration. The mixture was incubated for 20 min at 40°C, and the absorbance was measured at 660 nm. One unit of enzyme activity was expressed as giving an absorbance of 1/4000 under the above conditions[16]. Samples (100 ml) of grown *Bacillus pumilus* culture broth were removed every 4 h from 500-liter fermenter and centrifuged at 4000 rpm for 10 min. The supernatant was used as the source of extracellular protease.

Purification of protease

All purification steps were performed at 4°C. The bacterial supernatant was centrifuged at 10,000 rpm for 30 min, and then the pellet was discarded. All the supernatant was passed. The filtrate was used for purification of protease as follows.

The culture liquid was centrifuged at 10,000 rpm for 15 min, and then ammonium sulfate was added into the supernatant to a final concentration of 40% to precipitate the others. After centrifugation, ammonium sulfate was increased to 60% to get crude protease. The precipitate was dialyzed at 4°C in the dialyzer of a molecular weight cut-off of 8,000-12,000 Da. The dialyzate was freeze-dried.

The crude enzyme powder was dissolved in 50 mM Tris-HCl buffer (pH 7.3). The enzyme solution was applied to a DEAE-Cellulose-52 column (16 mm × 400 mm) equilibrated with 50 mM Tris-HCl buffer (pH 7.3) and the protease was eluted from the column with a linear gradient of 0-500 mM NaCl, 50 mM Tris-HCl buffer (pH 7.3). The fractions with protease activity were collected and dialyzed against 10 mM Tris-HCl buffer (pH 7.3) at 4°C. The active traction as a single peak from the column was obtained.

Molecular weight determination

The molecular weight of purified protease was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with phosphorylase B (molecular weight 97,400 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da) and lysozyme (14,400 Da) as standard proteins.

Effect of temperature and pH on protease activity

To investigate the influence of temperature on protease activity, the proteolytic activity of the protease was measured at the temperature range of 30-70°C, at pH 7.0 for 60 min. Prior to the assays, the enzyme sample, substrate solution and 50 mM sodium phosphate buffer (pH 7.0) were pre-incubated at desired temperature for 2 min. The effect of pH on protease activity was investigated using 50 mM of sodium phosphate buffer of pH 5.5-9.5 at the optimum temperature previously determined for 30 min. Substrate solution of azocasein was prepared in the respective buffers. All of the assay procedure was the same as described above.

Influence of temperature on protease stability

The protease was analyzed by incubating it in a 50 mM sodium phosphate buffer (pH 7.0) at different temperature (30°C, 35°C, 40°C, 45°C, 50°C, and 60°C). Samples were removed at different interval times (0.5 h, 1.0 h, 1.5 h, 2.0 h and 2.5 h), and the residual proteolytic activity was determined by azocasein hydrolyzed respectively.

Effect of metal ions and inhibitors on protease activity

To investigate the effect of metal ions on protease activity, the following chemicals were tested at the concentration of 1 mM: Fe^{2+}, Na^{+}, K^{+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Sn^{2+} and Cd^{2+}. The protease samples were incubated for 15 min in the presence of several surfactants (0.1% Glycerin, 0.1% tween 80, 0.1% Span 20 and 0.1% tween 20) at the room temperature and in the presence of various chemical denaturants (0.5%-1.0% SDS, 1-6 M urea, 0.5-6 M GdnHCl and 80-100 mM dithiothreitol) at 40°C, and then the residual protease activity was determined as above.

Analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The samples were boiled for 5 min with the presence of 1% SDS, 80 mM dithiothreitol, 100 mM Tris-HCl buffer (pH 7.3) and 15% glycerol, and then loaded onto a 10% SDS polyacrylamide gel. Electrophoresis was performed at a constant current (20 mA) at 4°C. After electrophoresis, the gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in methanol-acetic acid-water solution (30:10:60, v/v/v) and destained in isopropyl acetic acid-water solution (1:8, v/v/v).

Results and Discussion

Purification and molecular weight determination of *Bacillus pumilus* CN8 protease

The purification of *Bacillus pumilus* CN8 was achieved by ammonium sulphate precipitation, dialysis, and DEAE-Cellulose-52 ion-exchange chromatography during ion-exchange chromatography. The enzyme was purified as a single peak from gel-filtration chromatography. Electropherogram of the enzyme obtained by SDS-PAGE revealed only a single band, corresponding to a molecular mass of 96.92 kDa (Fig. 1). These results indicated that the protease is a monomeric enzyme and its molecular mass was greater than that of most alkaline proteases ranged from 15 kDa to 30 kDa, which produced by several bacteria including *Bacillus*[3]. Furthermore, after a series of purified operations, *Bacillus pumilus* CN8 protease was purified 13.2-fold over the culture supernatant and was recovered in 35.0% yield (Table 1).

Effect of temperature on the protease activity

The effect of temperature on the proteolytic activity from 30°C to 70°C in a 50 mM sodium phosphate buffer (pH 7.0) was shown in Fig. 2. It can be observed that the protease showed high activity at 40°C-60°C, and the maximum activity was obtained at 42°C. At the temperature range (40-60°C), the protease showed more than 90% of its optimal activity, while at temperatures above 60°C or below 40°C, the decrease in activity was sudden. The optimal temperature for a protease of *Bacillus cereus* was reported to be 40°C which is similar to the present study[17]. In addition, the protease could offer potential application in many fields due to its good action at a broad temperature range.

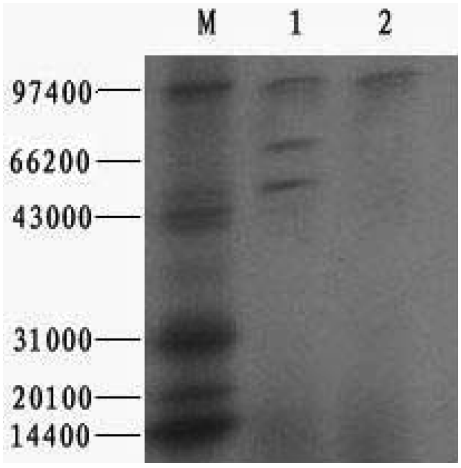

Fig. 1. Electropherogram of protease Lane M: molecular standard low-range markers: phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa); Lane 1: Crude enzyme. Lane 2: purified enzyme.

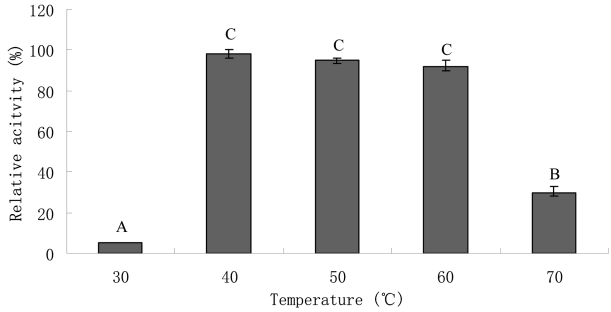

Fig. 2. Effect of different temperatures on the activity of *Bacillus pumilus* CN8 protease. Values are means of three independent experiments. Bars labeled with different capital letters are significantly different ($P < 0.05$).

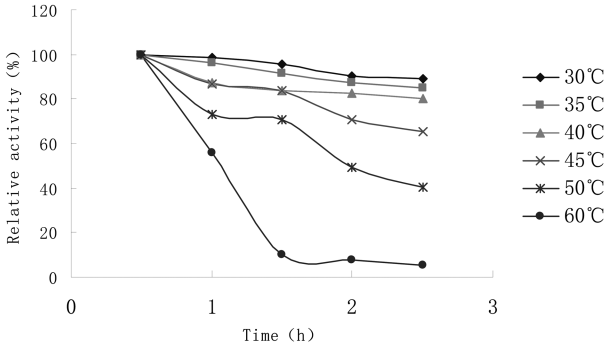

Fig. 3. Effect of different temperatures on the stability of *Bacillus pumilus* CN8 protease. Values are means of three independent experiments.

Fig. 3 represented the effect of temperature on enzyme stability of *Bacillus pumilus* CN8 protease. The results indicated that *Bacillus pumilus* CN8 protease was very stable at a suitable temperature which was from 30-40°C. It was

Table 1. Purification of protease from *Bacillus pumilus* CN8

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery of enzyme (%)	Purification multiple
Culture supernatant	43103	828.59	52.02	100	1
After 40% $(NH_4)_2SO_4$ supernatant	35603	295.98	120.29	82.6	2.3
After 60% $(NH_4)_2SO_4$ precipitation	26077	45.99	567.01	60.5	10.9
DEAE-Cellulose - 52	15068	21.97	685.84	35.0	13.2

stable at 30°C and 35°C for 2.5 h. However, after incubated at 50°C for 2 h or at 60°C for 1 h, protease remained an activity of only 50% compared with the activity obtained at optimum temperature.

Effect of pH on the protease activity

The activity of the protease was determined at 40°C after the enzyme was incubated at pH 5.5~9.5 at room temperature for 30 min. The results were shown in Fig. 4. The purified protease showed high activity at pH 6.5-9.5 wherein the protease retained 72% of its optimal activity at least, and the highest activity of the protease was obtained at pH 7.3. However, it retained only 50% of its optimum activity at pH 5.5. The effect of pH on the protease activity implied that the protease was a kind of neutral protease and was active in a broad range of pH values ranging from 6.5 to 9.5. These results are similar to the protease of *Bacillus polymyxa* B-17[18], *Bacillus subtilus*[19] and *Bacillus coagulans*[20].

Effect of metal ions on protease activity

Many metal ions often influence the activity of proteases[21,22]. The effect of 10 kinds of metal ions on the protease activity was tested (Fig. 5).

Effect of different metal ions on *Bacillus pumilus* CN8 protease stability was various. The addition of K^+ increased protease activity by 116% of the control. However, the enzyme activity could be strongly inhibited by Cu^{2+}, Mn^{2+}, Sn^{2+} and Cd^{2+} in 1 mmol/l concentration. Other ions such as Zn^{2+}, Fe^{2+}, Na^+, Ca^{2+}, Mg^{2+} had no significant activation or inhibition in 1 mmol/l concentration to the protease. Jang et al. reported that thermicin, a novel subtilisin-like serine protease from *Thermoanaerobacter yonseiensis* KB-1, was inhibited by $CuSO_4$, and $ZnCl_2$[22]. Consequently, K^+ had a protective effect on the enzyme activity. And Cu^{2+}, Mn^{2+}, Sn^{2+}, Cd^{2+} had a strong inhibitory effect on the protease activity. The reason for the metal ion inactivation may be oxidation reaction or complex formation occurred between the metal-sensitive amino acid residues and the metal ion[23].

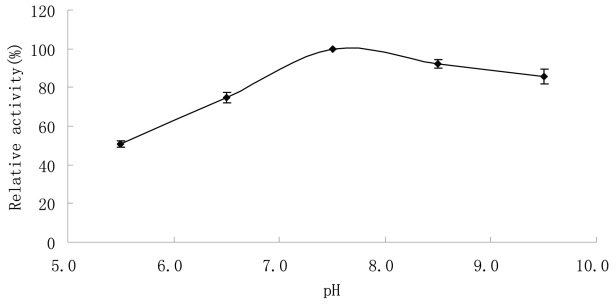

Fig. 4. Effect of different pH values on the activity of *Bacillus pumilus* CN8 protease. Values are means of three independent experiments.

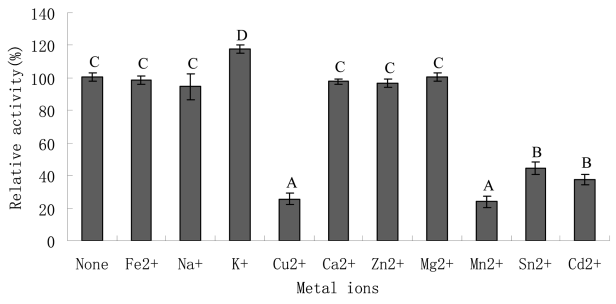

Fig. 5. Effect of different metal in 1 mmol/l concentration on *Bacillus pumilus* CN8 protease activity. Values are means of three independent experiments. Bars labeled with different capital letters are significantly different ($P < 0.05$).

The resistance to surface active agent

Fig. 6 shows the effect of different surfactants on the activity of *Bacillus pumilus* CN8 protease. The enzyme activity could be obviously inhibited by Glycerin and retained only 75% of its activity. Tween 80 had little impact on enzyme activity. It retained 98% of its activity in the presence of tween 80. Similar results are reported about the proteases from *Bacillus mojavensis* A21 with 1% tween 80[24]. Conversely, Span 20 and tween 20 could promote the protease activity. From those data, *Bacillus pumilus* CN8 protease seems to be more stable against these surface active agents except Glycerin.

The resistance to chemical denaturants

The activity of the *Bacillus pumilus* CN8 protease in the presence of different concentrations of chemical denaturants was examined (Table 2). The protease shows significant resistance to all the denaturing reagents at 40°C. In addition, an enhancement in the enzymatic activity was observed at a high concentration of 1% SDS, 4-6 M urea and 0.5-1 M GdnHCl. The *Bacillus pumilus* CN8 protease was resistant to chemical denaturants including SDS, dithiothreitol, urea and GdnHCl, which indicated that the protease can keep the

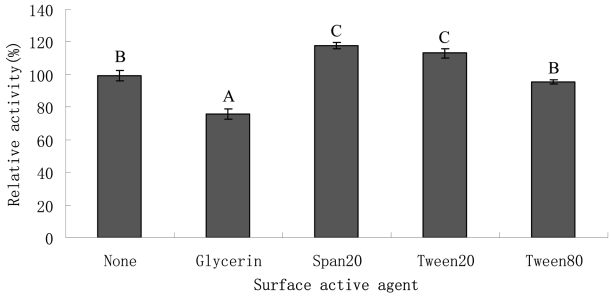

Fig. 6. Effect of different surfactants on the activity of *Bacillus pumilus* CN8 protease. Values are means of three independent experiments. Bars labeled with different capital letters are significantly different ($P < 0.05$).

Table 2. The effect of chemical denaturants on protease activity of *Bacillus pumilus* CN8

Reagent	Concentration	Relative activity (%)
None		100
SDS	0.5%	100
	1.0%	153
GdnHCl	0.5 mol/l	120
	1.0 mol/l	139
	6.0 mol/l	16
Urea	1.0 mol/l	92
	4.0 mol/l	105
	6.0 mol/l	120
Dithiothreitol	80 mmol/l	100
	100 mmol/l	100

structure stability. The results shows that *Bacillus pumilus* CN8 protease was highly stable in the presence of the strong anionic surfactant (1% SDS) at 40ºC for l0min.

Conclusions

The present investigation relates to the purification and characterization of *Bacillus pumilus* CN8 protease and effects of different factors on its protease activity and stability. *Bacillus pumilus* CN8 protease was a neutral protease and performed the maximal activity at pH 7.3 and 42ºC. It produced highly efficient enzyme-degrading of porcine hemoglobin, thereby can utilize porcine hemoglobin. The separation steps were performed after cultivation as following: the culture liquid was centrifuged at 10000 rpm for 15 min, and then ammonium sulfate was added into the supernatant to a final concentration of 40% to precipitate the others. After centrifugation, ammonium sulfate is increased to 60% to get crude protease. *Bacillus pumilus* CN8 protease is purified to homogeneity from liquor by ultrafiltration, ammonium sulfate precipitation, dialysis, DEAE-Cellulose-52 gel filtration. The specific activity of the enzyme is 686.66 U/mg. Purification times and recovery rate of enzyme are 13.2 and 35.0% respectively. The *Bacillus pumilus* CN8 which belonged to the topical neutral protease showed a higher enzyme activity when the pH was between 6.5 and 9.5 wherein the protease retained 72% of its optimal activity at least. The molecular weight of the purified enzyme is estimated to be 96.92 kDa. The *Bacillus pumilus* CN8 protease was stable when the temperature was between 30ºC and 40ºC. K^+ could slightly increase the protease activity. The enzyme activity could be heavily inhibited by Cu^{2+}, Mn^{2+}, Sn^{2+}, Cd^{2+}. Other ions such as Zn^{2+}, Fe^{2+}, Na^+, Ca^{2+}, Mg^{2+} had no significant activation or inhibition of the protease.

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