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Characterization of *Bacillus cereus* SH-7 Extracellular Protease

Hak Kyu Yi, Young Jin Chun[1], and Han Bok Kim*

Department of Life Science, Hoseo University, Asan-Si, ChungNam 336-795;
[1]College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea

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An extracellular endopeptidase from *Bacillus cereus* SH-7 was purified to homogeneity. The protease was most active at pH 8 and 40°C, respectively. The molecular mass of the protease was 40 kDa on SDS-PAGE, and 120 kDa by gel filtration, suggesting that the native enzyme is composed of three homogeneous subunits. The K_m and V_{max} values of the protease for *N*-succinyl-$(Ala)_2$-Pro-Phe-*p*-nitroanilide were 11.11 mM and 170 nmol/mg of protein/min, respectively. The protease was also identified as a metalloprotease. The bioactivity of the SH-7 protease will need further study in the future.

Key words: *Bacillus cereus*, metalloprotease, extracellular protease

Proteases are produced by a variety of microorganism, and they are classified as serine (9, 14, 17), cysteine (19), aspartate (18), or metallo (17) proteases according to the amino acids in their active sites. Proteases can be also classified as acidic (18), neutral (24, 28) or alkaline (9, 14, 17) ones according to their optimal pH. Some proteases are active even at high temperatures (10).

Proteases are important enzymes which are widely used in the pharmaceutical and food processing industries. One of the *Bacillus* proteases, nattokinase, is known to have fibrinolytic activity (13, 26), and *Serratia* protease an anti-inflammatory effect (23). Also, subtilisin Carlsberg (8), BPN' (17) from *Bacillus* strains are used as laundry detergents.

Many microorganisms, *Virio* (5), *Serratia* (15), *Pseudomonas* (7), and *Brevibacterium* (22) secrete proteases. Many *Bacillus* strains also produce extracellular proteases (10-12, 21, 22, 24, 28). It is convenient to study extracellular protease since its purification is relatively simple.

A *Bacillus* sp. strain producing an extracellular protease was previously isolated from Korean soybean paste, and identified as *B. cereus* SH-7 (11) through a series of physiological, biochemical tests, and the automatic identification systems, VITEK and MIDI (25). Fatty acids of 15:0 iso and 17:0 iso are major ones in the strain, which is typical for the *B. cereus* strain isolated from Korean sea water (12). Some *B. cereus* strains have characteristics of producing hemolysin (2), enterotoxin (1), phospholipase C (27), or β-lactamase (20).

There is also a report that *B. cereus* protease is composed of a single subunit of 29 kDa (24). However, homo- or heterogeneous multi-subunit structure of the *B. cereus* protease has not yet been reported. The *B. cereus* SH-7 protease was purified and characterized in this study. The secretory endopeptidase has molecular mass of 120 kDa and three homogeneous subunits, which is distinct from previously reported proteases (24, 28).

* To whom correspondence should be addressed.
(Tel) 82-418-540-5334; (Fax) 82-418-548-6231
(E-mail) hbkim@dogsuri.hoseo.ac.kr

Materials and Methods

Strain and culture

Bacillus cereus SH-7 producing a secretory protease was used in this study (11). The strain was cultured in Luria-Bertani (LB) media or LB containing 1.5% skim milk at 37°C.

Protease purification

B. cereus SH-7 was cultured in LB, 37°C for 12 h. It was diluted 100-fold in new LB, and cultured again for 18 h. The cell culture was centrifuged at 10,000 × *g* for 10 min. The supernatant containing the extracellular protease was then collected, and precipitated with 50-80% ammonium sulfate. The precipitate was centrifuged at 10,000 × g for 20 min, and then dialyzed in 20 mM sodium phosphate buffer (pH 7.5) overnight. Ion exchange chromatography by DEAE Sepharose CL-6B equilibrated with a 20 mM citric acid-sodium

phosphate dibasic buffer (pH 5.5) was performed on the dialysate. A column was eluted by a linear gradient of 0~1 M NaCl at a rate of 0.5 ml/min. The collected and concentrated enzyme solution was then applied to a Sephadex G-150 gel filtration column equilibrated with a 20 mM sodium phosphate buffer (pH 7.0) with a flow rate of 1.05 ml/min. LB agar media with skim milk were used for the detection of the fractions containing a protease.

Determination of molecular mass

The molecular mass of the native protease was determined, using a Sephadex G-150 gel filtration column calibrated with several standard markers. SDS-PAGE was used for characterizing the sizes and types of subunits in the purified protease.

Protease assay and kinetic studies

The protease activity was determined using a chromogenic oligopeptide as a substrate (*N*-succinyl-L-$(Ala)_2$-L-Pro-L-Phe-*p*-nitroanilide) in 20 mM Tris-HCl (pH 8.5). The mixture containing the purified protease and substrate was incubated at 37°C for 1 h. The liberated *p*-nitroanilide was determined spectrophotometrically at 410 nm. One unit was defined as the amount of enzyme that released one μmol of *p*-nitroaniline (ε_{410}=8480 $M^{-1}cm^{-1}$) (6). Protein concentation was determined by the Bradford method (3). Various concentrations of the protease substrate were added into the basic enzyme reaction mixture. A Lineweaver-Burk plot was drawn for the determination of the K_m and V_{max}.

Effect of protease inhibitor

The purified enzyme and various inhibitors (0.1 mM) were added into an enzyme reaction mixture containing the protease substrate, and the residual activity was then determined.

Effect of pH and temperature on protease activity

The optimal pH and temperature of the protease activity and its stability were determined in a Britton-Robinson buffer at various temperatures and pH.

Results and Discusssion

Purification of *B. cereus* SH-7 protease

Cells cultured for 18 h were used for the protease purification since the protease activity reached the highest level at the culture time (11).

Since the SH-7 strain secretes its protease, the supernatant of this strain's cell culture was, therefore, used for the protease isolation. The supernatant containing the protease was then precipitated by 50~80% ammonium sulfate.

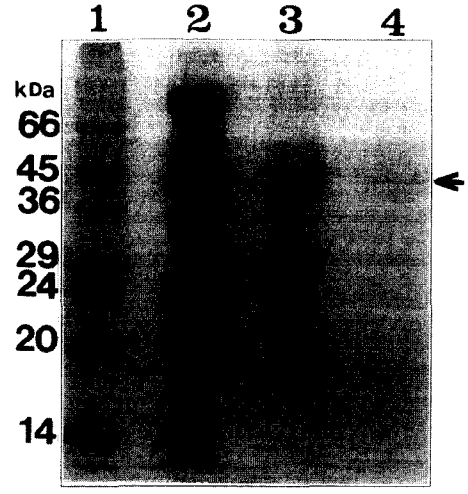

Fig. 1. SDS-PAGE gel of purified SH-7 protease. Lane 1; Marker proteins, Lane 2; proteins precipitated by ammonium sulfate, Lane 3; proteins eluted by DEAE exchange chromatography, Lane 4; protease eluted by Gel Filtration chromatography. The arrow in Lane 4 indicates the position of the SH-7 protease.

For further purification of the protease, ion exchange and gel filtration chromatography were performed. The fractions containing the protease were easily identified by their halo formations on LB agar containing skim milk, since the protease has caseinolytic activity. The extracellular protease was so stable that chromatography could be performed at room temperature using DEAE exchange chromatography. The protease eluted around 0.8 M NaCl by a gradient elution. The purity of each elution during the purification steps was determined by SDS-PAGE (Fig. 1). The active fraction in gel filtration chromatography exhibited a single band of 40 kDa by SDS-PAGE, which is almost a pure state (Fig. 1).

Molecular mass of the protease

A single band on SDS-PAGE does not necessarily indicate the existence of a monomer in the native protein, since there can be homogeneous multi-subunits. The apparent molecular mass of the native protease of strain SH-7 was determined to be 120 kDa by gel filtration chromatography calibrated with standard markers (Fig. 2), while the native protease showed only a single band of 40 kDa by SDS-PAGE (Fig. 1), suggesting that the protease is composed of three identical subunits. The Trimer structure of the extracellular protease in *B. cereus* is not yet reported to the best of our knowledge. It might be postulated that the hydrophobic domain of secretory protease facilitates oligomeric structure formation.

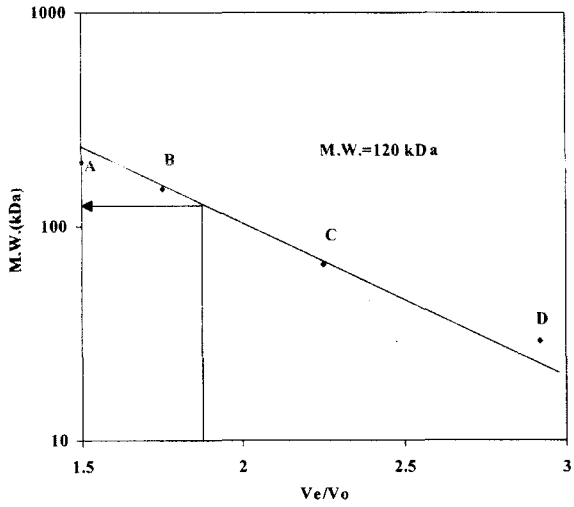

Fig. 2. Determination of the molecular mass of the protease from *Bacillus cereus* SH-7 by Sephadex G-150. A; β-amylase (200 kDa), B; alcohol dehydrogenase (150 kDa), C; albumin bovine serum (66 kDa), D; carbonic anhydrase (29 kDa)

Sierecka (24) reported that a virulent strain of *B. cereus* had an extracellular protease (29 kDa) in the native enzyme, and Wetmore *et al* (28) reported that the protease (36 kDa) purified from *B. cereus* DSM 3101 was a mature form by SDS-PAGE. The molecular mass of the SH-7 protease was clearly larger than the marker protein (36 kDa) (Fig. 1). Thus, it would seem that the protease (40 kDa) of strain SH-7 in this study is a novel form, and that different strains of *B. cereus* produce various sizes of extracellular proteases. The *B. cereus* group is known to be very diverse.

Protease activity and kinetic study

The purified protease showed clear proteolytic activity when *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide was used as a substrate (Fig. 3). Varying concentrations of the substrate in the enzyme reaction allowed the determination of the K_m and V_{max} using a Lineweaver-Burk plot. The K_m was 11.11 mM, and the V_{max} 170 nmol/mg of protein/min (Fig. 3). If different substrates are used, the K_m values may change, and could be lower in some cases.

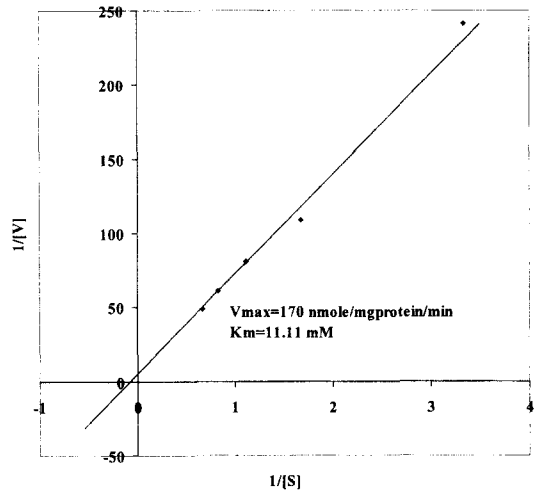

Fig. 3. The kinetics of SH-7 protease activity.

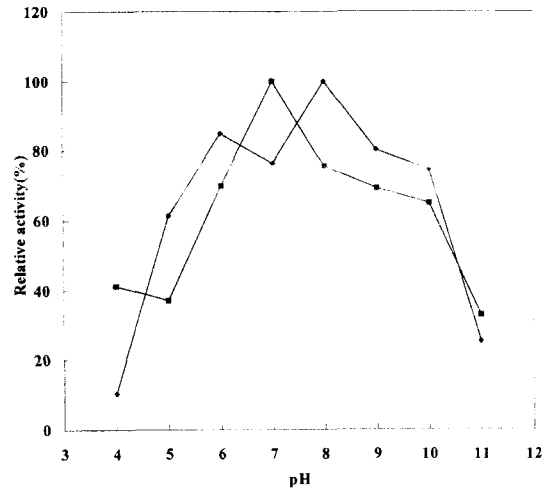

Fig. 4. Effect of pH on SH-7 protease activity. (◆-◆); activity, (■-■); stability

The protease seemed to be an endopeptidase, considering that the amino- and carboxy-terminals of the substrate were blocked. So far extracellular proteases purified from *B. cereus* strains are known to be of the endopeptidase type (28). Some *Bacillus* strains produce exopeptidases such as aminopeptidases (4, 21). The protein is generally cleaved by the endopeptidase, and then the exopeptidase degrades the oligo-peptide into amino acids. Whether or not *B. cereus* SH-7 produces exopeptidase can be determined by using

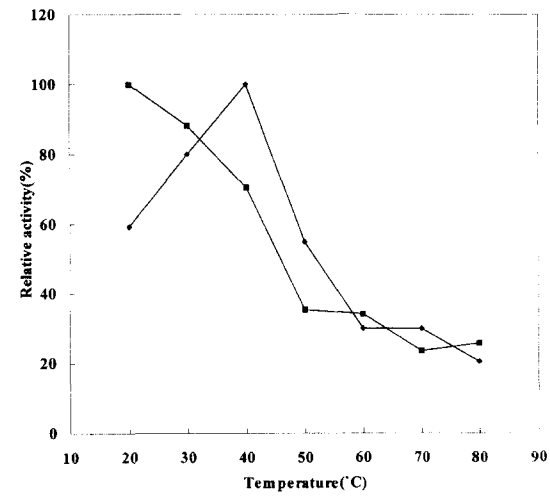

Fig. 5. Effect of temperature on SH-7 protease activity. (◆-◆); activity, (■-■); stability

Table 1. Effect of inhibitors on SH-7 protease activity

Inhibitors	Concn (mM)	Relative activity (%)
None	0.1	100
EDTA	0.1	21
PMSF	0.1	112
Benzamidine	0.1	112
Leupeptin	0.1	105

substrates specific for aminopeptidase or carboxylpeptidase.

The purified SH-7 protease was strongly inhibited by 0.1 mM EDTA, suggesting that it is a metalloprotease (Table 1). However, PMSF (serine protease inhibitor), benzamidine (trypsin, plasmin, thrombin inhibitor), leupeptin (some cysteine and serine proteases inhibitor) did not inhibit SH-7 protease activity (Table 1). Thus it would seem that *Bacillus cereus* SH-7 extracellular protease is not a serine protease.

There is a previous report that the extracellular protease of a strain of *B. cereus* has a pro-sequence in addition to a mature form (16, 28). The pro-sequence is finally removed from the mature protease once it is transported out of a cell. The pro-sequence was recently discovered to have an intramolecular chaperone to catalyze the folding of its associated protein (16). Further study on the possible pro-sequence from *B. cereus* SH-7 will contribute to an elucidation of the pro-sequence-assisted folding mechanism. It seems that the removal of the pro-sequence from *B. cereus* SH-7 allows trimer assembly out of the cell.

Effect of pH and temperature

The purified protease was optimal at pH 8, and most stable at pH 7. Thus, the protease is a neutral one (Fig. 4). It was also shown that at least 50% of the maximum activity in the stable protease was within the range of pH 5.5~10 (Fig. 4). The extracellular proteases isolated from other *B. cereus* strains were also neutral in their optimal pHs. Since the protease isolated in this study showed considerable activity even in alkaline pH, it could be used in laundry detergents.

Some proteases produced from thermophilic *Bacillus* are heat-resistant. The protease of *B. cereus* SH-7 was most active at 40°C, stable at 20°C, and became rapidly inactive above 50°C. The protease exhibited 60% of maximum protease activity at 20°C and was also most stable at 20°C (Fig. 5).

Many microbial proteases can be used in the pharmaceutical area. For instance, the *Bacillus* protease, nattokinase, is known to have fibrinolytic activity *in vivo* (26). *Serratia marcescens* produces an extracellular protease which is used widely as an anti-inflammatory drug (23). The mechanism of these effects in the organism are still unknown. The bioactivity of the *B. cereus* SH-7 protease should be studied further now that it has been purified and its biochemical characterization has been completed. It will be interesting to investigate the possible fibrinolytic activity of *B. cereus* SH-7 protease when it is taken orally.

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