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Purification and Some Properties of a Thermostable Protease, BSP2, Produced from Bacillus *stearothermophilus No. 2*

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A thermophilic bacteria, *Bacillus stearothermophilus* No. 2 isolated from sewage sludge compost, was found to produce a thermostable neutral protease (BSP2). The BSP2 was purified from culture broth by fractionation with cold acetone and subsequent column chromatographies. The purified BSP2 gave a single band on SDS — PAGE and its molecular weight was estimated to be 35kDa. The purified protease was optimally active at pH 7.5 — 8 and showed stability at pH range of 6 to 11.5. The thermostability of the BSP2 was markedly improved under the presence of Ca^{2+} (5mM $CaCl_2$) with 60% residual activity after 80min heat-treatment at 90 ℃ and the optimum proteolytic activity was at 75 ℃ Its proteolytic activity was almost completely inactivated by metal chelaters such as EDTA and EGTA, and not by IAA or PMSF. The NH,-terminal amino acid sequence of the BSP2 showed high homology with those of thermostable neutral proteases from reffered *Bacillus* strains reported so far.

INTRODUCTION

Thermostable protease have been the subject of intensive research in the past years because of their potent application in various industrial processes as well as an interest in biochemical study. Considerable efforts are continuing to isolate new novel thermophiles as sources of thermostable proteases.

Thermolysin, which is well-known thermostable neutral protease produced by mesothermophilic *Bacillus thermoproteolyticus*, is of industrial important and specially has been studied in detail (Endo, 1962; Kester ans Matthews, 1977; Latt *et al.*, 1969; Morihara and Tsuzuki, 1970; Stauffer, 1971). The primary and tertialy structures of thermolysin have been revealed (Titani *et al.*, 1972; Matthews *et al.*, 1972a; Mattews *et al.*, 1972b). Recently, highly thermostable neutral proteases from *Bacillus stearothermophilus* and *Bacillus caldolyticus* have been reported and their primaly structures showed extremely high homology with those of thermolysin (Kubo *et al.*, 1983b; Heinen and Heinen, 1972; Kubo and Imanaka, 1988; Van Der Burg *et al.*, 1991). From our previous work, twelve strains of thermophilic bacteria were isolated from sewage sludge compost. Among them, *Bacillus stearothermophilus No. 2* (strain No. 2) was found to produce an extracellular protease (Fujio and Kume, 1991).

This paper deals with the purification and the properties of the protease produced by *Bacillus stearothermophilus No. 2* isolated from compost.

MATERIALS AND METHODS

Microorganism and cultivation media

Bacillus stearothennophilus No. 2 (strain No. 2), formerly isolated and identified

(Fujio and Kume, 1991) was used in this study. The strain No. 2 was incubated on agar slants (nutrient broth, pH 7.5) at 60 ℃ for 24h. The culture was inoculated in liquid medium (0.5% yeast extract, 0.1% polypeptone, 0.1% casein peptone, 0.1% geratin, 0.1% KH_2PO_4, dissolved with distilled water ans adjusted pH to 7.5) in a 500ml flask to prepare the inoculum for protease production. The cultivation was done at 60 ℃ for 2 days. For protease production, 10% of the seed culture was inoculated into the medium composed of 1.5% Bacto peptone, 0.5% casein peptone, 0.3% yeast extract, 0.2% NaCl, 0.1% K_2HPO_4 in distilled water with an initial pH of 7.5. The cultivation was carried out at 60 ℃ for 24 hours with a jar fermentor (21, working volume 1.51). The bacterial cells were then removed from the culture broth by centrifugation, and supernatant n-as used as the enzyme source.

Assay of protease activity

The proteolytic activity was assayed by the method of Long *et al.* (1981) with slight modification. Azocasein (Sigma) was used as substrate at a concentration of 1% in 0.05M potassium phosphate buffer (pH 7.5). A 0.5ml of enzyme solution n-as added to 1ml of substrate solution and the mixture was incubated at 70 ℃ for 10 min. The reaction was terminated by the addition of 10% trichloroacetic acid. After standing at 4 ℃ for 10 nun, the mixture was filtrated and 1ml of 0.4M NaOH was added to 1ml of the filtrate. The absorbance of this mixture was measured at 440nm. One unit of protease activity was defined as amount of enzyme that gave an increase in absorbance of 0.1 at 440nm in above condition.

Purification of the protease

Next 4 steps of purifications were performed at a cold room at 4C. **Step 1:** acetone precipitation: two volumes of cold acetone (- 20°C) was slowly added into the culture supernatant with gentle stirring. The mixture was allowed to stand overnight in a cold room, afterwhich the precipiate foemed was collected by centrifugation. The precipitate n-as then dissolved with a portion of 10mM potassium phosphate buffer (pH 6.8) and dialyzed against the same buffer. **Step 2:** hydroxylapatite chromatography: the dialysate (crude enzyme solution) was charged on a column of hydroxylapatite (Nacalai Tesque) which previously equilibrated with 10mM potassium phosphate buffer (pH 6.8). After the column was washed with the same buffer, the enzyme adsorbed was eluted with 80mM potassium phosphate buffer (pH 6.8). The fractions with major protease activity were collected and dialyzed against 50mM Tris-HCl (pH 7.5). **Step** 3: DEAE-Sephadex A-50 chromatography: the dialysate was then charged on a column of DEAE-Sephadex A-50 (Pharmacia) which previously equilibrated with 50mM Tris-HCl (pH 7.5). After the column was washed with the same buffer, the enzyme adsorbed was eluted with a linear gradient of 0-0.5M NaCl in the same buffer. The fractions with major protease activity n-ere collected again and concentrated by ultrafiltration with a membrane filter (10kDa molecular weight cut-off, Advantec). **Step** 4: Sephadex G − 75 superfine gel filtration: the concentrate was then charged on a column of Sephadex G-75 superfine (Pharmacia) which had been equilibrated with 50mM potassium phosphate buffer (pH 7.5). The protease active fractions through the column was collected as the purified BSP2 protease. The BSP2 was stored in the frozen state at − 20 ℃ until use.

Protein determination

Protein concentration was determined using a protein assay kit (Bio-Rad Chemical

Division, Calif, USA) with bovine serum albumin as a standard protein.

SDS-polyacrylamide gel electrophoresis

Homogeneity of BSP2 was examined by SDS-PAGE on a slab gel containing 12.5% of polyacrylamide and 5% of 2 − mercaptoethanol (Nacalai Tesque). Molecular weight of BSP2 was also determined by comparing its mobility with following standard proteins. The standard proteins used were phospholylase b (94kDa), bovine serum albumin (67kDa), carbonic anhydrase (30kDa), trypsin inhibitor (20.1kDa) and α-lactalbumin (14.4kDa). The proteins were stained with coomassie brilliant blue R − 250.

Effect of inhibitors on the proteolytic activity of the protease

The effect of some protease inhibitors on the activity of the protease was examined in the following method. The BSP2 solution was mixed with each inhibitor solution at a final concentration of 1mM. After standing at room temperature for 30min, remaining activity was assayed.

Hydrolysis of various proteins

Various proteins including casein, lysozyme, haemoglobin, collagen, elastin, keratin and fibrinogen were used as substrate. The reaction was carried out at 70°C for 20min. The amount of extricated amino acids by the reaction was determined by the method of Yemm and Cocking (15) as glycine standard.

NH,-terminal amino acid sequence of the protease

The NH_2-terminal amino acid sequence of the purified enzyme was determined using an Applied Biosystems (Foster City, Calif, USA) Protein/Peptide Sequencer, Model 473A. Identification of phenylthiohydantoin derivatives of amino acids was performed in an Applied Biosystem PTH analyser model 610A system.

RESULTS AND DISCUSSION

Purification of the protease

The BSP2 from the strain No. 2 was purified as shown in Table 1 which summarizes major purification steps. The specific activity of purified BSP2 was 46.3 folds higher than that of culture supernatant with a recovery of 8.2%. Figure 1 shows the result of final gel chromatogram (step 4). Figure 2 shows the homogeneity of the BSP2 by SDS-PAGE wirh some standard proteins. From Fig. 2, the BSP2 gave a molecular weight of 35kDa.

Table 1. Summary of purification.

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Culture supernatant	1930	695	272.2	2.5	100
Acetone precipitation	150	537	108.3	5.0	77.3
Hydroxylapatite	76	195	16.9	11.6	28.1
DEAE-Sephadex A-50	69	157	2.3	67.5	22.6
Sephadex G-75 superfine	17	57	0.5	115.8	8.2

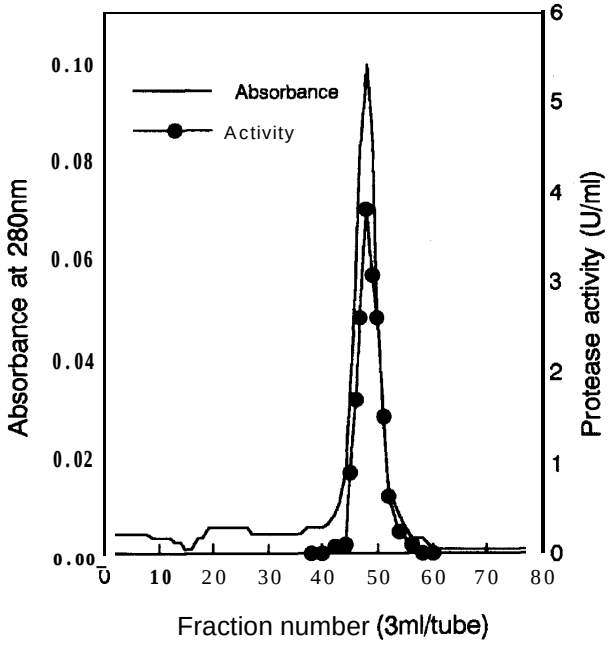

Fig. 1. Elution profile of Sephadex G-75 superfine.

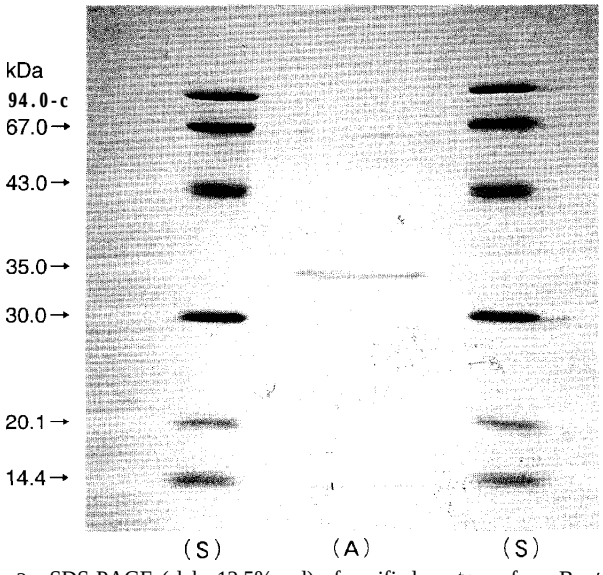

Fig. 2. SDS-PAGE (slab, 12.5% gel) of purified protease from B. *stearothermophilus No. 2.*

A; Purified protease

S; Standard proteins (Electrophoresis caliburation kit, Pharmacia). 94.0 kDa (phosphorylase b), 67.0 kDa (bovine serum albumin), 43.0 kDa (ovalbumin), 30.0 kDa (carbonic anhydrase), 20.1 kDa (trypsin inhibitor), 14.4 kDa (α-lactalbumin)

Effect of pH on the proteolytic activity and stability of the protease

Figure 3 shows the optimum pH for proteolytic activity of thr BSP2. The optimum pH was **7.5 – 8.0** and no change observed in the presence or absence of $CaCl_2$. Figure 4 shows that the BSP2 was almost, stable in the pH range of 6– 11.5 after treatment at 4°C for 24 hours. The pH stability was not affected whether with or without $CaCl_2$.

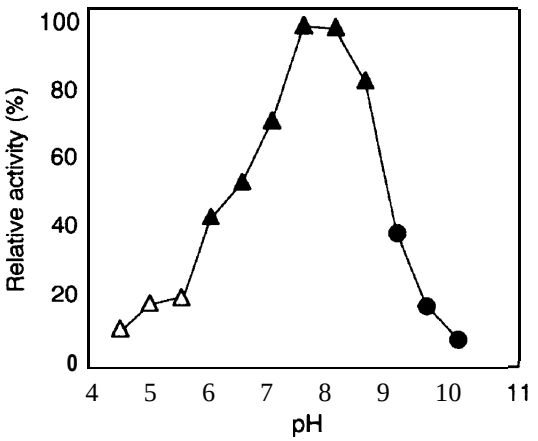

Fig. 3. Effect of pH on the proteolytic activity of protease from *B. stearothermophilus* No. 2. The reaction was carried out at 70°C in the following buffers; acetate (A), phosphate (A) and carbonate (●).

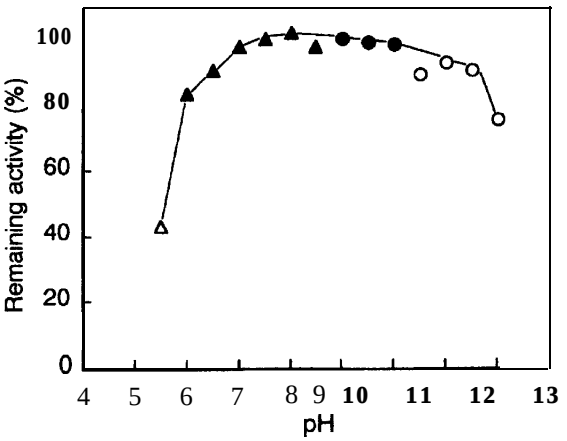

Fig. 4. Effect of pH on the stability of protease from *B. stearothermophilus* No. 2.
The pH treatment was carried out at 4°C for 24hr in the following buffers: acetate (A), phosphate (A), carbonate (●) and sodium phosphate/NaOH(○).

Effect of temperature on the proteolytic activity and stability of the protease

Figure 5 shows the effect of temperature on the proteolytic activity of the BSP2. The optimum temperature for the proteolytic activity was 75°C in the presence of 5mM $CaCl_2$. Figure 6 shows the temperature dependency of the BSP2. The effect of temperature on the stability of the BSP2 was examined at 90°C in the presence or absence of $CaCl_2$. In the presence of $CaCl_2$, optimum temperature was estimated to be at 90C, while the optimum temperature was sifted down to 70°C in absence of $CaCl_2$. Even after the treatment at 90°C for 80min, more than 60% of original activity was remained in the presence of $CaCl_2$. As in many reports related to metalloñprotease, calcium ion seemed markedly to increase the stability of the protease.

Effect of inhibitors on the proteolytic activity of the protease

Table 2 shows the effect of inhibitors on the proteolytic activity of BSP2. Metal chelaters such as EDTA or EGTA almost completely inactivated the activity of BSP2. Although IAA showed some effect on the activity, this might be the result of which IAA reacted with side groups of a number of amino acids (Gundlach et al., 1959). Therefore, NSP2 from strain No. 2 may be classified to a metallo-protease.

In view point of the effects of inhibitors and optimum pH, the BSP2 from strain No.2 is clearly classified as an neutral protease. Most of microbial neutral proteases are metal cherater sensitive and showed no or a little inhibition toward other inhibitors (McConn et *al.*, 1964).

Hydrolysis of various proteins

Table 3 shows the hydrolysis of various proteins by BSP2 including some insoluble

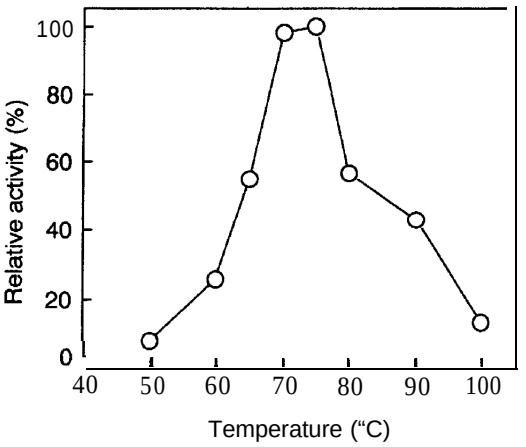

Fig. 5. Effect of temperature on the proteolytic activity of protease from B. *stearothermophilus No.* **2.** in the presence of 5mM $CaCl_2$.
The enzyme activity was assayed at the indicated temperature in the presence of 5mM $CaCl_2$.

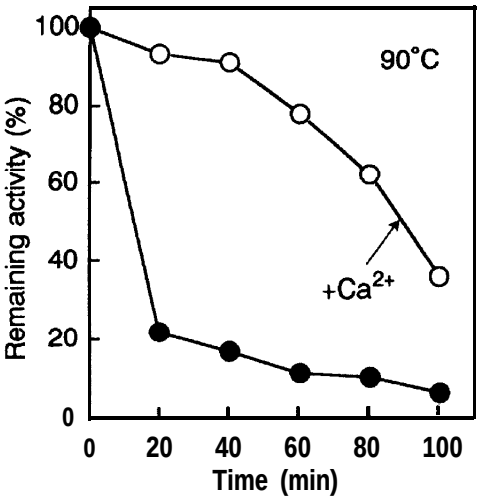

Fig. 6. Effect of temperature on the stability of protease from *B. stearothermophilus No.* **2.** After incubation at 90 ℃ for the indicated times in the presence (○) or absence (●) of 5mM $CaCl_2$, the remaining activity was assayed.

Table 2. Effect of inhibitor on the proteolytic activity protease.

Inhibitor (mM)	Remaining activity (%)
None	100
EDTA(l)	7
EGTA(1)	0
IAA(1)	58
PMSF(l)	97

EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis (β-aminoethylether) -N, N, N', N'-tetraacetic acid; IAA, iodoacetic acid; PMSF, phenylmethylsulphonyl fluoride

proteins. Among them, the highest degree of hydrolysis was observed in casein. In the insoluble proteins, such as collagen, elastin and haemoglobin (the latter precipitated rapidly at 70°C) were hydrolyzed a little amount while keratin and fibrinogen were not hydrolyzed at all. Although the strain No.2 was isolated from sewage sludge compost, this neutral protease, BSP2, may be a little contribution to decompose sewage sludge.

NH,-terminal amino acid sequence of the protease

Table 3. Hydrolysis of various proteins by protease.

Substrate	Proteolytic activity (μg glycine/ml)
Casein	53.2
Lysozyme	31.5
Haemoglobin	10.5
Collagen	9.6
Elastin	9.3
Keratin	0
Fibrinogen	0

The reaction was carried out at 70°C for 20min.

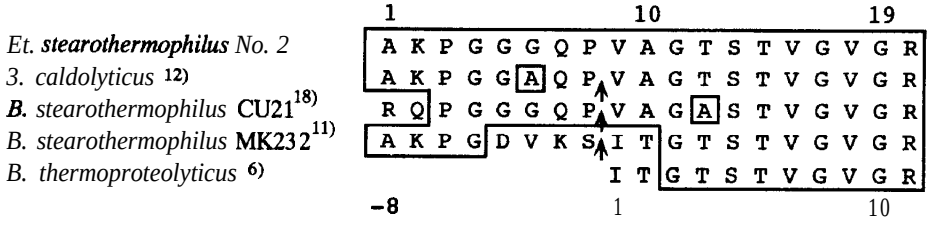

Fig. 7. NH,-terminal amino acid sequence of protease (BSP2) from strain No. 2 and its comparison with neutral proteases from *Bacillus strains.*
Amino acids identical to those of BSP2 from strain No. 2 (*Bacillus stearothermophilus No. 2)* are boxed. Amino acid sequences of proteases from *Bacillus caldolyticus, Bacillus stearothermophilus* CLJ21 and MK232 were deduced from their nucleotide sequences. Arrows indicate the maturation sites.

The NH,-terminal amino acid sequence of BSP2 was determined. Figure 7 shows the comparison with those of thermostable neutral proteases from reffered *Bacillus* strains (Titani et al., 1972; Kubo and Imanaka, 1988a; Van Der Burg et *al.*, 1991; Takagi et al., 1985). The amino acid sequence of the first 19 residues from NH,-terminus of protease from the strain No. 2 showed extremely high homology with those from − 8 to 11 of the neutral proteases from refered *Bacillus* strains. Although the significance of the excess eight amino acid residues of the BSP2 was not clear, they seemed not to participate in the function of catalysis of the protease.

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