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Purification and characterization of two extracellular alkaline proteases from a newly isolated obligate alkalophilic *Bacillus sphaericus*

J Singh, RM Vohra and DK Sahoo

Biochemical Engineering Research and Process Development Centre, Institute of Microbial Technology, Sector-39A, Chandigarh 160036, India

Two novel extracellular serine proteases were purified to homogeneity from the cell-free culture filtrate of an obligate alkalophilic *Bacillus sphaericus* by a combination of ultrafiltration, ammonium sulfate precipitation and chromatographic methods. The enzymes showed similar substrate specificities, but differed in hydrophobicity and molecular mass. Protease A was a monomeric protease with a relative molecular mass (M_r) of 28.7 kDa, whereas protease B, with a M_r of 68.0 kDa, apparently consisted of smaller subunits. The purified protease A had a specific activity on hemoglobin of 5.1 U/mg protein compared to 40.9 U/mg protein in the case of protease B. Both proteases were most active on SAAPF-pNa, a substrate for chymotrypsin-like serine proteases. However, the K_m values of these two proteases on SAAPF-pNa were higher than that for α-chymotrypsin, indicating a lower affinity of proteases A and B for this substrate compared to chymotrypsin. Unlike other *Bacillus* serine proteases, neither protease A nor B stained with Coomasie blue R-250, even with loading of a large amount of protein, and they stained poorly with the silver staining method. However, NH_2-terminal amino acid sequencing of protease B revealed a high similarity with subtilisin Carlsberg (67% homology). Almost total inhibition of both proteases by PMSF, but very little/no inhibition by trypsin and chymotrypsin inhibitors (TPCK and TLCK) or thiol reagents (PCMB and iodoacetic acid), further supported the view that the enzyme belonged to the serine protease family. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 387–393.

Keywords: alkalophilic *Bacillus*; serine protease; properties; substrate specificity

Introduction

Proteolytic enzymes from the genus *Bacillus* are so far the most important group of commercial enzymes with applications in the food, pharmaceutical and detergent industries and their use as an active ingredient in detergents is the single largest use of industrial enzymes [2,12]. Alkaline proteases are added to detergents to facilitate the release of proteinaceous materials in stains such as those due to milk, grime and blood. Some detergents contain chlorine bleaches, and water supplies are chlorinated in many parts of the world, including India. In order to prevent chlorine from attacking and inactivating enzymes, especially under alkaline conditions, chlorine bleach scavengers are included in detergent compositions as a part of the enzyme-stabilizing system [7]. The food processing industry has come to rely on improved detergent efficiency in their cleaning programmes. Protease incompatibility with chlorine is cited as one of the reasons why its use is not so widespread as a part of cleaning agents in the food processing industry [10]. Previously, during a search for novel alkaline proteases, we isolated an obligate alkalophilic *Bacillus sphaericus* from alkaline Himalayan soils. Alkaline proteases from this organism showed chlorine compatibility, in addition to their potential as additives in laundry detergents and in recovering silver from used X-ray films [23]. *B. sphaericus* is generally studied for its ability to produce a proteinaceous parasporal body, which is toxic to the larvae of a number of mosquito species that are responsible for transmission of human and animal diseases, but there are very few reports on alkaline protease production by this organism.

In this study, we purified and characterized two novel extracellular proteases from this obligate alkalophile and obtained additional data on molecular, biochemical and catalytic properties.

Correspondence: Dr DK Sahoo, Biochemical Engineering Research and Process Development Centre, Institute of Microbial Technology, Sector-39A, Chandigarh 160036, India
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Materials and methods

Materials

Protease inhibitors, substrates such as azocasein, azoalbumin, hemoglobin, casein, BSA and oligopeptidyl-*p*-nitroanilides, gel electrophoresis and isoelectric focusing (IEF) reagents, SDS and sodium benzene sulfonate were purchased from Sigma (St. Louis, MO, USA). Sodium perborate was obtained from Fluka Chemie (Buchs, Switzerland). All other chemicals were of analytical reagent grade.

Microorganism and culture conditions

An obligate alkalophilic *B. sphaericus* was isolated from alkaline soils of the Himalayas (India) and deposited at the Microbial Type Culture Collection Centre and Gene Bank, Institute of Microbial Technology, Chandigarh, India, under accession no. MTCC-B-0014, as described elsewhere [23]. The medium for pre-inoculum, inoculum as well as enzyme production contained (g/l): glucose, 10.0; biopeptone, 5.0; yeast extract, 5.0; KH_2PO_4, 1.0;

$MgSO_4 \cdot 7H_2O$, 0.2; and Na_2CO_3, 10.0. Biopeptone (Hi Media, Bombay, India) was a combination of mixed peptones derived from fresh meat and milk proteins. The solution of Na_2CO_3 was sterilized separately and then added to the medium.

Pre-inocula were prepared by transferring a loop-full of freshly prepared culture from skim milk agar plates [23] into 250-ml flasks containing 50 ml of medium and incubating the flasks at 30°C and 200 rpm on a rotary shaker for 24–27 h. Flasks (500 ml) containing 100 ml of medium were inoculated with 10% v/v of this pre-inoculum and incubated at 200 rpm and 30°C for 12 h to serve as inoculum for the fermenter. A Chemap fermenter (Chemap, Mannedorf, Switzerland), with a 5 l working volume, was used for protease production. After inoculating the fermenter with 500 ml of inoculum, it was aerated at 1.0 vol/vol/min (vvm) and agitated at 300 rpm.

Purification of extracellular proteases

After 24 h of fermentation, cells were harvested by centrifugation (at 5000×*g* for 30 min). The supernatant was concentrated 10-fold by tangential-flow ultrafiltration using a Pellicon system (Millipore, Bedford, MA, USA) equipped with a 10-kDa cut-off membrane. Retentate from this step was further concentrated by ammonium sulfate precipitation at 70% saturation. This precipitate was dissolved in Tris–HCl buffer (0.025 M, pH 9.0) and dialyzed overnight against the same buffer.

Hydrophobic interaction chromatography (HIC)

The dialyzed enzyme preparation was applied to a phenyl agarose column (1.0×10 cm) connected to a Gradifrac protein purification system (Pharmacia, Uppsala, Sweden). Tris–HCl buffer (0.05 M, pH 9.0), containing 1.0 mol/l NaCl, was used for loading the sample at a flow rate of 0.5 ml/min and 8.0-ml fractions were collected. The column was washed with the same buffer and bound proteins were eluted by applying a linear gradient of 0–50% v/v ethylene glycol at a flow rate of 1.0 ml/min and monitored at 280 nm.

Ion exchange chromatography

Active fraction(s) of protease from the phenyl agarose column were pooled and loaded onto a Q Sepharose (Pharmacia) ion exchange column (2.5×10.0 cm) equilibrated with Tris–HCl (0.05 M, pH 9.0) buffer and connected to a Gradifrac protein purification system. The buffer used was Tris–HCl (0.05 M, pH 9.0) at a flow rate of 0.5 ml/min. After washing the column with the same buffer, bound proteins were eluted by applying a linear gradient of 0–1.0 M NaCl in buffer at a flow rate of 1.0 ml/min. Fractions (8.0 ml) were collected and analyzed for enzyme activity and purity.

Protease assays

Proteolytic activity was estimated at pH 10.0 with azocasein as substrate [20]. Incubation was at 37°C for 30 min. Activity on azoalbumin was assayed following the same protocol as for azocasein. Protease activity, as U/ml, was calculated as the increase in absorbance at 420 nm (1 cm path length) caused by 1.0 ml of enzyme per hour of incubation under the assay condition.

Activity on casein, hemoglobin and BSA was assayed at pH 10.0 and 37°C. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 micromole of tyrosine equivalent per minute.

The purified enzyme was tested for its ability to hydrolyze synthetic peptide substrates conjugated to *p*-nitroaniline (pNA). *N*-Suc–Ala–Ala–Pro–Phe–pNA, a substrate for chymotrypsin [5]; *N*-BZ-D,L-Arg–pNA, a substrate for trypsin [6]; *N*-Cbz–Ala–Aal–Leu–pNA and *N*-Cbz–Gly–Gly–Leu–pNA, substrates for subtilisin [17]; *N*-Suc–Ala–Ala–Ala–pNA, *N*-Suc–Gly–Gly–Phe–pNA, *N*-Suc–Phe–Ala–pNA and *N*-Acetyl–L–Ala–pNA were used. Stock solutions (5 mM) of the substrates were prepared in dimethyl sulfoxide (DMSO). Substrate (25 μl) was added to 465 μl of buffer (0.05 M glycine–NaOH, pH 10.0), mixed and pre-incubated at 37°C for 5 min. The reaction was started by addition of 10 μl of enzyme solution. After 10 min at 37°C, the reaction was stopped by addition of 500 μl of 10% (w/v) citric acid and the absorbance was measured at 410 nm. One unit of *p*-nitroanilide-hydrolyzing activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitroaniline per minute at 37°C and pH 10.0.

The K_m values of purified proteases A and B were determined from the initial rate of hydrolysis (incubation time 5 min) of *N*-Suc–Ala–Ala–Pro–Phe–pNA at pH 10.0 (0.05 M glycine–NaOH buffer) and 37°C using an Eadie–Hofstee plot.

Protein estimation

Protein was quantified by a modified Lowry method [21] using bovine serum albumin (Sigma) as standard.

Effects of chemical reagents (class-specific protease inhibitors)

Enzyme solution (10 μl) and 470 μl of assay buffer containing various chemical reagents at a given concentration were mixed and pre-incubated at 37°C for 2 h. This was followed by addition of 20 μl of 5% azocasein. Proteolytic activity was measured as described above. The following chemical reagents were used: phenylmethylsulfonyl fluoride (PMSF), *p*-chloromercuric benzoic acid (PCMB), tosyl L-lysine chloromethyl ketone (TLCK), tosyl L-phenylalanine chloromethyl ketone (TPCK), ethylenediaminetetraacetic acid (EDTA), *N*-CBZ-L-phenylalanine chloromethyl ketone (ZPCK), iodoacetic acid, 1,10-phenanthrolein and benzamidine (all from Sigma).

Gel electrophoresis

Discontinuous gel electrophoresis was carried out using a modified method of Laemmli [16] with the SE 600 electrophoresis system of Hoefer (San Francisco, CA, USA). Stacking was done in a gel of 4% T and the resolving gel was 12.5% T. Stacking was done at 6 mA and the resolving gel was operated at 9 mA using a power supply from Hoefer (USA; PS 500 XT). For denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), samples were denatured by boiling them with sample buffer for 90 s before loading them onto the gel. The relative molecular mass (M_r) was calculated using protein standards (low range molecular weight markers) from Sigma. Protein bands were visualized by silver staining [4].

IEF

IEF was performed in a Multiphore II system of Pharmacia according to the protocol recommended by the manufacture [3]. In gel casting solutions, ampholytes (Sigma) were incorporated at a concentration of 2%. Sodium hydroxide (20 mM) was used as the

upper chamber buffer (cathode) and 0.085% phosphoric acid as the lower chamber buffer (anode). After IEF was over, the gel was fixed in a mixture of 5% sulfosalicylic acid and 10% trichloroacetic acid for 30 min. Protein bands were visualized by silver staining [4]. The p*I* of the protein was calculated from a standard curve of marker proteins, supplied by Pharmacia.

NH_2-terminal amino acid sequencing

Purified protease B was electrophoresed on 10% SDS-PAGE and then transferred to a PVDF membrane (Millipore) with CAPS buffer, pH 11.0, according to the protocol of Matsudaira [19]. Transfer of the protein was verified by staining with amido black. A strip containing the protease was cut out and incubated in acetonitrile for 12 h and it was then loaded on protein sequencer. Edman degradation was performed on an Applied Biosystems model 476A protein sequencer equipped with an online PTH analyzer.

Results and discussion

In our screening programme, we had isolated an obligate alkalophilic *B. sphaericus* from an alkaline Himalayan soil sample. The crude alkaline protease from this organism was optimally active at pH 10.5 and 50–55°C, and the presence of $CaCl_2$ increased the thermostability of the enzyme [23]

Purification

Two extracellular proteases, A and B, were purified from the culture supernatant of *B. sphaericus* to apparent homogeneity as per the purification scheme presented in Figure 1. HIC of the dialysate on phenyl agarose resolved it into two fractions: HIC-1, which did not bind to the matrix even in the presence of 1 M NaCl in buffer, and of HIC-2, which bound to phenyl agarose. The binding of HIC-2 to phenyl agarose was so strong that it could not be eluted even when NaCl was removed from the buffer and elution was attempted using 50% v/v ethylene glycol. When these two fractions were applied separately to a Q Sepharose column, both were found to bind to this matrix (Figure 2). Protease B, representing the major protease activity, was obtained with an overall recovery of almost 52%, compared with 1.0% recovery of protease A (Table 1).

Molecular mass and isoelectric point

Proteases A and B were desalted and concentrated. When these two fractions were loaded onto SDS-PAGE, no protein band was

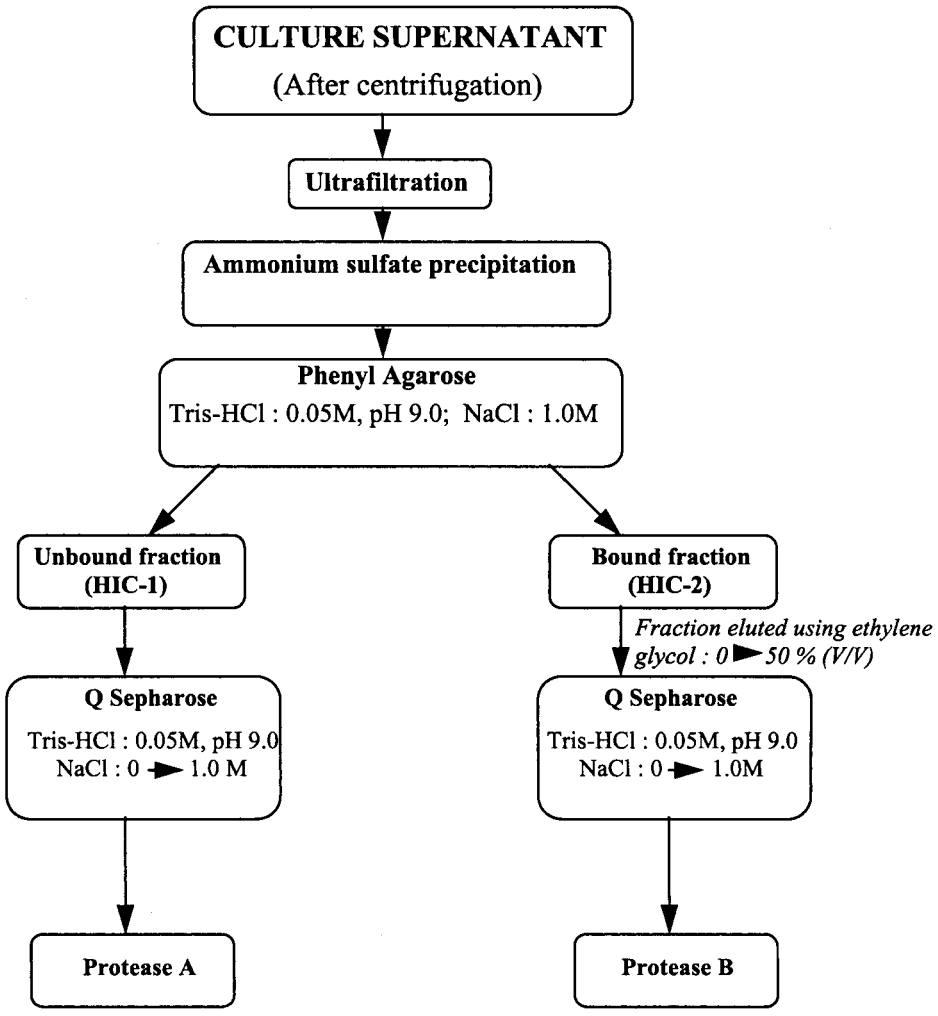

Figure 1 Purification scheme for two alkaline proteases from *B. sphaericus*.

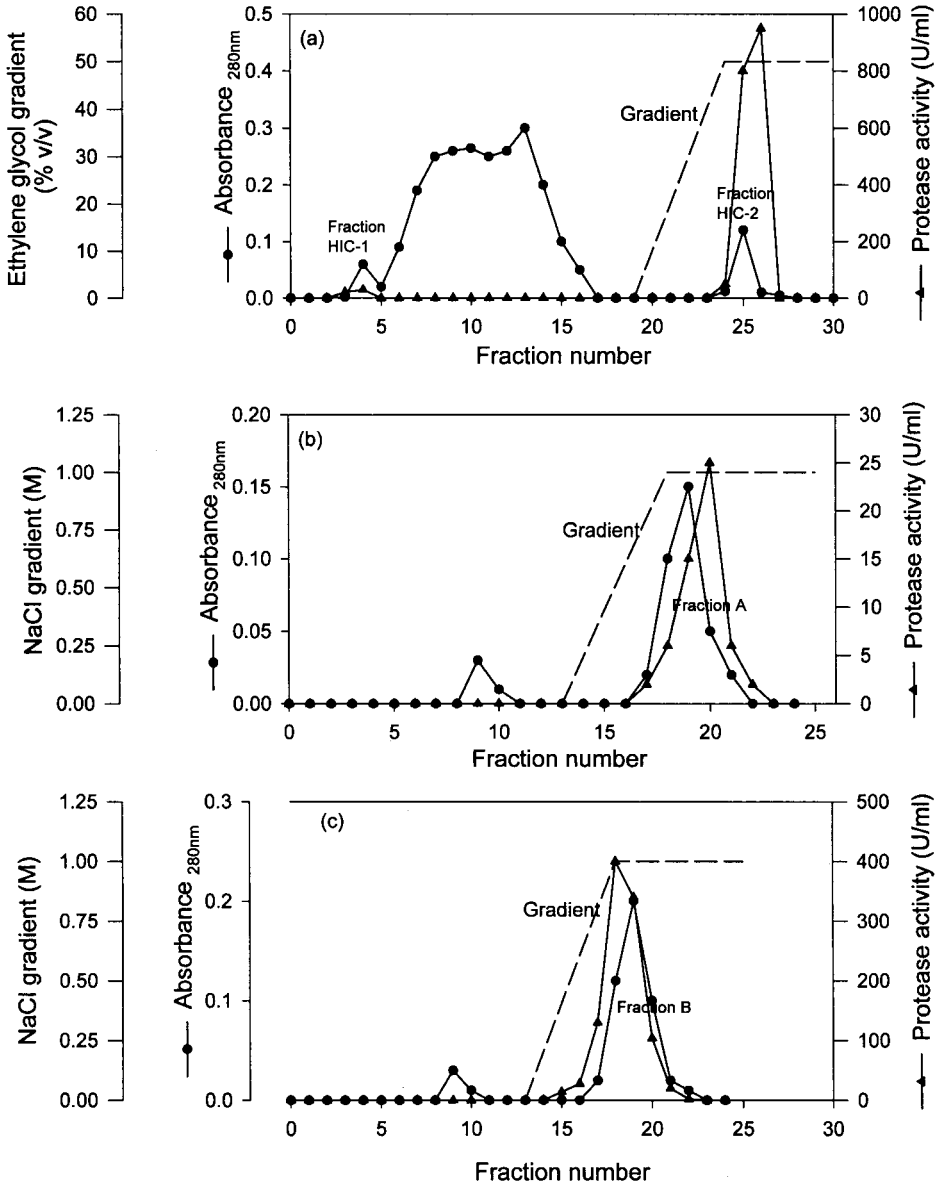

Figure 2 Typical chromatograms obtained at different steps of purification. (a) HIC of ammonium sulfate precipitate fraction prepared from ultrafiltration retentate of *B. sphaericus* culture supernatant. Active fraction(s) of protease from phenyl agarose column was pooled as fractions HIC-1 and HIC-2 and loaded into Q Sepharose column. (b) Ion exchange chromatography of HIC-1 and (c) ion exchange chromatography of HIC-2.

observed. However, addition of PMSF to the proteases before boiling the solution with sample buffer led to visualization of protein bands. Unlike other *Bacillus* serine proteases, neither protease A nor B stained with Coomasie blue R-250 even on loading a large amount of protein, and they stained poorly with the silver staining method [9,15,22]. Similar poor staining properties were reported for a chymotrypsin-like serine protease from a *Streptomyces* sp. [30]. The purified proteases appeared to be homogeneous on SDS-PAGE (Figure 3). Protease A (with M_r of 28.7 kDa) was found to be a monomeric enzyme, whereas protease B (with M_r of 68 kDa) apparently consisted of smaller subunits. Previously, Yoshida *et al* [32] reported a single protease of molecular mass of 27 kDa from a strain of *B. sphaericus*. However, multimolecular forms of proteases have been reported from other strains of *Bacillus* [15,33] and from *Arthrobacter nicotinae* [24]. The isoelectric point of the major protease, i.e., protease B, was 8.6. Since the best performance of proteases is observed at pH values near their p*I*, a high p*I* is a desirable characteristic of proteases that are to be used in a laundry detergent or other applications at high alkaline pH [12,29].

Substrate specificity

The specificity of proteases A and B towards various substrates was examined, and results are shown in Table 2. Although proteases A and B differed in hydrophobicity and molecular mass, they showed similar substrate specificity. Among natural substrates, hemoglobin was most efficiently hydrolyzed by both proteases, followed by casein and BSA. Both azoalbumin and azocasein were hydrolyzed by proteases A and B. The substrate specificity of the enzymes was investigated in more detail with a number of synthetic substrates

Table 1 Purification of extracellular proteases A and B from *B. sphaericus*

	Total protein (mg)	Total protease activity (U)	Species activity (U/mg protein)	Activity recovery (%)
Protease A				
Cell-free culture supernatant*	308.5	47200	153	100.0
Ultrafiltrated retentate*	117.2	44300	378	93.9
Ammonium sulphate precipitated enzyme solution*	16.9	44000	2601	93.2
Phenyl agarose	2.2	692	315	1.5
Q Sepharose	1.44	471	327	1.0
Protease B				
Cell-free culture supernatant*	308.5	47200	153	100.0
Ultrafiltered retentate*	117.2	44300	378	93.9
Ammonium sulphate precipitated enzyme solution*	16.9	44000	2601	93.2
Phenyl agarose	10.3	33120	3228	70.2
Q Sepharose	7.23	24653	3408	52.2

*The enzyme activity on cell-free culture supernatant, ultrafiltered retentate and ammonium sulfate-precipitated enzyme corresponds to the sum of both the protease activities.

(*p*-nitroanilides). The substrates were blocked at the N-terminus and bore chromophoric groups at the C-terminus. Studies on specificity of the proteolytic activity on amino acid and peptide *p*-nitroanilides showed both proteases to be most active on *N*-Suc–Ala–Ala–Pro–Phe–pNA, a substrate for chymotrypsin-like serine proteases. In contrast, each showed less hydrolytic activity towards *N*-Cbz–Ala–Ala–Leu–pNa and *N*-Cbz–Gly–Gly–Leu–pNa, which are good substrates for subtilisins. *N*-Cbz–Ala–Ala–Leu–pNa, a common substrate for subtilisins, is hydrolyzed with high specificity by extracellular serine proteases from different microorganisms [26]. Chymotrypsin substrates other than *N*-Suc–Ala–Ala–Pro–Phe–pNA such as *N*-Suc–Gly–Gly–Phe–pNA and *N*-Suc–Phe–Ala–pNA were not hydrolyzed by protease A, and protease B exhibited very low catalytic activity toward *N*-Suc–Gly–Gly–Phe–pNA. The K_m values of proteases A and B for the chymotrypsin substrate, *N*-Suc–Ala–Ala–Pro–Phe–pNA, were determined to be 9.6×10^{-5} M and 11.6×10^{-5} M, respectively. Since the K_m of α-chymotrypsin for SAAAPF-pNA is 4.3×10^{-5} M under similar condition [25], proteases A and B were found to have lower affinity for this substrate than for chymotrypsin. The elastage substrate *N*-Suc–Ala–Ala–Ala–pNA was weakly hydrolyzed and *N*-Bz-D,L-Arg–pNA, a substrate specific for trypsin, was not hydrolyzed at all by these two proteases.

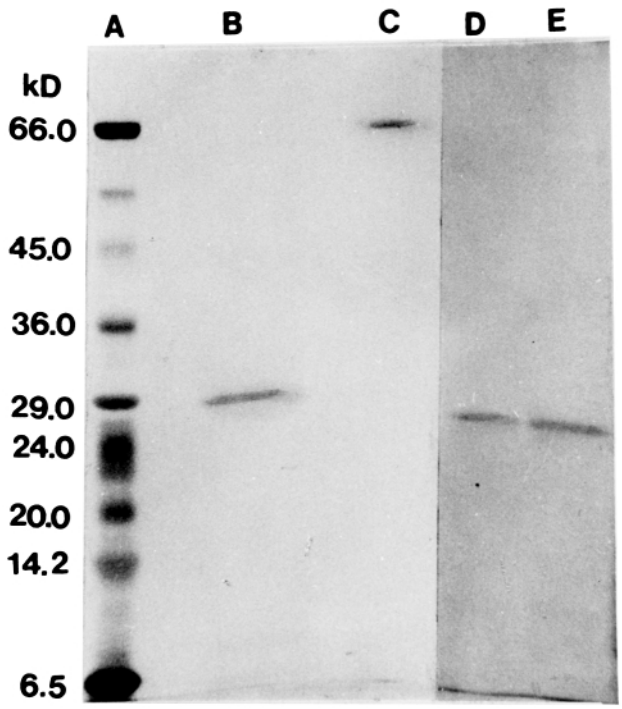

Figure 3 SDS-PAGE of purified proteases A and B from *B. sphaericus*. Lane A: molecular weight marker proteins. Lane B: protease B (boiled). Lane C: protease B (nonboiled). Lane D: protease A (nonboiled). Lane E: protease A (boiled).

NH_2-terminal amino acid sequence analysis

The NH_2 terminal amino acid sequence of the first 15 residues of *B. sphaericus* protease B was determined by Edman sequencing and compared with that of subtilisin Carlsberg, subtilisin BPN′; Elastage Ya-B, *Bacillus* proteases nos. AH-101 and 221, Savinase, *B. alcalophilus* and Espelase (Figure 4). Maximum similarity of this enzyme was with subtilisin Carlsberg (67% homology) [11]. However, there was 40% homology with subtilisin BPN′ [11]. The NH_2-terminal amino acid sequence of *B. sphaericus* protease B was also homologous with Elastage Ya-B (53%) [13], protease no. AH 101 (53%) [28], Espelase (53%) [31], protease no. 221 (48%) [28], *B. alcalophilus* protease (48%) [29], Savinase (48%) [31] and protease BYA (40%) [22].

Effects of chemical reagents (class-specific protease inhibitors)

The effect of a wide spectrum of inhibitors on proteases A and B was determined by measuring residual activity following 2 h pre-incubation of the enzymes with inhibitors (Table 3). The activity of both proteases was significantly inhibited by ZPCK, which inactivates subtilisins. Although there was 27.4% inhibition of protease A and 40.5% inhibition of protease B with the chelating agent EDTA, the other metalloprotease inhibitor 1,10-phenanthrolein did not influence enzyme activity. The basis for classification

Table 2 Substrate specificity of purified alkaline proteases A and B toward azoproteins, natural proteins and *p*-nitroanilide substrates

Substrate	Species activity (U*/mg)		% Relative activity[#]	
	Protease A	Protease B	Protease A	Protease B
(a) Azoproteins				
Azocasein	199	1865	93.0	93.0
Azoalbumin	214	2007	100.0	100.0
(b) Natural proteins				
BSA	2.0	14.5	38.9	35.4
Casein	2.9	23.3	57.0	56.9
Hemoglobin	5.1	40.9	100.0	100.0
(c) p-nitroanilide substrates				
N-Suc–Ala–Ala–Pro–Phe–pNA	1.62	12.20	100.0	100.0
N-Cbz–Ala–Ala–Leu–pNA	0.69	2.80	42.6	43.6
N-Cbz–Gly–Gly–Leu–pNA	0.11	1.15	6.8	9.4
N-Suc–Ala–Ala–Ala–pNA	0.07	0.48	4.3	4.0
N-Suc–Gly–Gly–Phe–pNA	nil	0.25		2.1
N-Suc–Phe–Ala–pNA	nil	nil		
N-Acetyl-L-Ala–pNA	nil	nil		
N-BZ-D,L-Arg–pNA	nil	nil		

*Experimental conditions and enzyme unit definitions for each substrate are given in text.
[#]% Relative activity = { [Species activity of protease A (or Species activity of protease B)] / [Species activity of protease A + Species activity of protease B] }.

of metalloproteases is the presence of a metal ion (usually zinc), which participates in catalysis, and 1,10-phenanthroline is usually preferred as a diagnostic indicator of metalloproteases because of its higher stability constant for zinc than for calcium [1]. There are

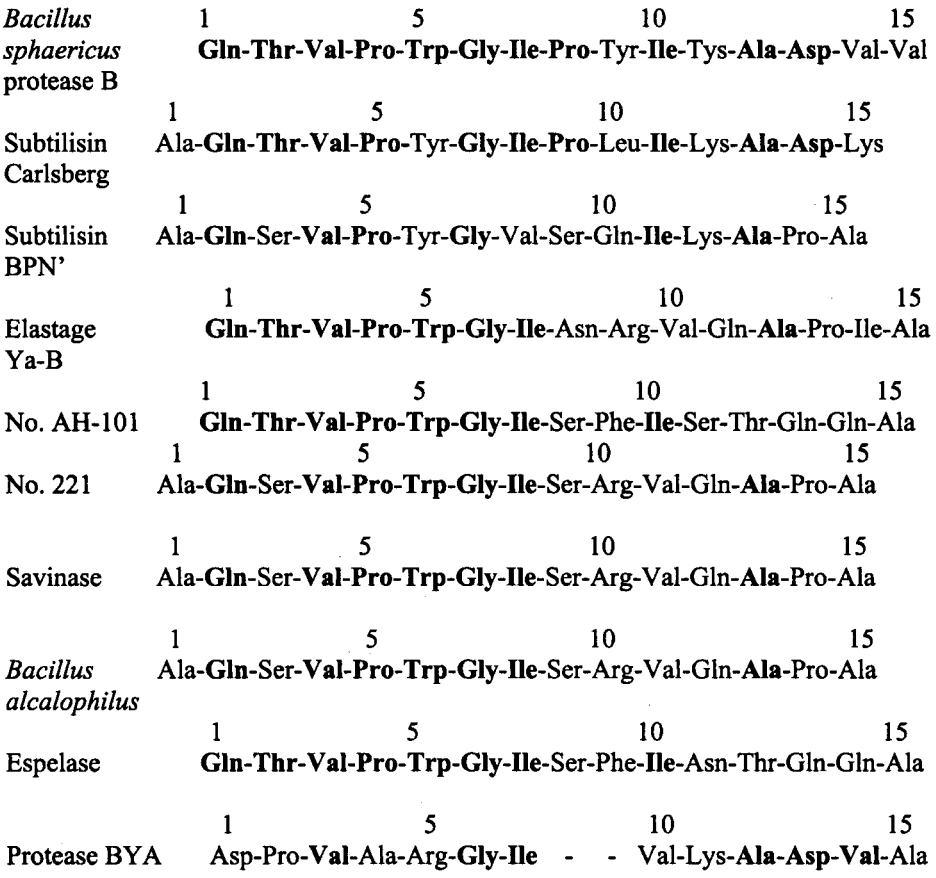

Figure 4 NH_2-terminal sequence of protease B purified from *B. sphaericus* and its comparison with sequences reported for subtilisin Carlsberg [11], subtilisin BPN′ [11], Elastage Ya-B [27], no. AH 101 [28], no. 221 [28], Savinase [23], *B. alcalophilus* [23], Espelase [23] and protease BYA [22]. Boldface type indicates residues that are the same.

Table 3 Effect of class-specific protease inhibitors on the activity of *B. sphaericus* protease

Inhibitor	Concentration (mM)	% Inhibition	
		Protease A	Protease B
Benzamidine	10	Nil	Nil
TLCK	1	Nil	Nil
1,10-Phenanthrolein	10	Nil	Nil
EDTA	10	27.4	40.5
PCMB	1	15.3	10.2
Iodoacetate	5	Nil	Nil
PMSF	5	98.5	92.8
TPCK	1	5.0	12.5
ZPCK	1	80.6	85.6

TLCK, tosyl L-lysine chloromethyl ketone; EDTA, ethylenediaminetetra-acetic acid; PCMB, *p*-chloromercuribenzoic acid; PMSF, phenylmethyl-sulphonyl fluoride; TPCK, tosyl L-phenylalanyl chloromethyl ketone; ZPCK, *N*-CBZ-L-phenylalanine chloromethyl ketone.

reports on serine proteases that are sensitive to EDTA [8,14,18,27] and EDTA is reported to completely inhibit the activity of a protease (called sfericase) from *B. sphaericus* [32]. There was little/no inhibition of activity of proteases A and B by TPCK, a specific inhibitor of chymotrypsin, or thiol reagents such as PCMB and iodoacetic acid. Almost total inhibition of proteases A and B by PMSF, an active site inhibitor of serine proteases, but not by TLCK, a specific inhibitor of trypsin, further supports the view that the enzymes belong to the serine protease family.

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