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Novel Alkaline- and Heat-Stable Serine Proteases from Alkalophilic *Bacillus* sp. Strain GX6638

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An alkalophilic *Bacillus* sp., strain GX6638 (ATCC 53278), was isolated from soil and shown to produce a minimum of three alkaline proteases. The proteases were purified by ion-exchange chromatography and were distinguishable by their isoelectric point, molecular weight, and electrophoretic mobility. Two of the proteases, AS and HS, which exhibited the greatest alkaline and thermal stability, were characterized further. Protease HS had an apparent molecular weight of 36,000 and an isoelectric point of ~4.2, whereas protease AS had a molecular weight of 27,500 and an isoelectric point of 5.2. Both enzymes had optimal proteolytic activities over a broad pH range (pH 8 to 12) and exhibited temperature optima of 65°C. Proteases HS and AS were further distinguished by their proteolytic activities, esterolytic activities, sensitivity to inhibitors, and their alkaline and thermal stability properties. Protease AS was extremely alkali stable, retaining 88% of initial activity at pH 12 over a 24-h incubation period at 25°C; protease HS exhibited similar alkaline stability properties to pH 11. In addition, protease HS had exceptional thermal stability properties. At pH 9.5 (0.1 M CAPS buffer, 5 mM EDTA), the enzyme had a half-life of more than 200 min at 50°C and 25 min at 60°C. At pH above 9.5, protease HS readily lost enzymatic activity even in the presence of exogenously supplied Ca^{2+}. In contrast, protease AS was more stable at pH above 9.5, and Ca^{2+} addition extended the half-life of the enzyme 10-fold at 60°C. The data presented here clearly indicate that these two alkaline proteases from *Bacillus* sp. strain GX6638 represent novel proteases that differ fundamentally from the proteases previously described for members of the genus *Bacillus*.

Alkaline proteases secreted by both neutralophilic and alkalophilic bacilli are of interest because they represent a major source of commercially produced proteolytic enzymes (1; 11, 16). These proteases exhibit optimal activity at pHs of 9 to 11 and are inactivated by serine active-site inhibitors, such as phenylmethylsulfonyl fluoride (PMSF) and diisopropylfluorophosphate. In general, these enzymes have molecular weights ranging from 20,000 to 30,000, are stabilized by Ca^{2+}, and have characteristically high isoelectric points (2, 11, 12, 16, 19, 23). Alkaline proteases, or subtilisins, secreted by neutralophilic *Bacillus* spp. are stable from pH 5 to 10 at low temperatures, but are readily inactivated at higher temperatures and alkalinities in the absence of Ca^{2+} (1, 2). The subtilisins have been divided into two groups based on differences in amino acid composition and immunological and kinetic properties (12). Recent results, however, suggest that these proteases possess identical amino acid residues in 63% of their primary sequences (17). Alkaline proteases from alkalophilic bacteria have been studied in less detail, yet many similarities exist between subtilisins and alkaline proteases from alkalophilic bacilli (11). However, serine proteases from alkalophiles have superior alkaline stability properties and extremely basic isoelectric points (2, 11).

In the present communication, we describe certain physical and biochemical properties of two alkaline proteases (AS and HS) produced by an alkalophilic microorganism, *Bacillus* sp. strain GX6638. We demonstrate that AS and HS from *Bacillus* sp. strain GX6638 share many properties common to the better-characterized *Bacillus* alkaline proteases. However, the alkaline proteases AS and HS differ from those previously characterized in that they are acidic proteins that fail to cross-react with polyvalent antiserum directed toward subtilisin BPN′ or bacillopeptidase F. In addition, both protease AS and protease HS have better alkaline and thermal stability than subtilisin Carlsberg. We suggest that the two alkaline proteases (AS and HS) isolated from *Bacillus* sp. strain GX6638 represent novel proteases that differ fundamentally from the alkaline proteases previously isolated from members of the genus *Bacillus*.

MATERIALS AND METHODS

Media and culture conditions. Isolates were routinely cultivated on PSC solid agar medium which contains (per liter) 10 g of Bacto-peptone (Difco Laboratories), 10 g of Bacto-yeast extract (Difco), 5 to 10 g of potato starch, 1 g of dibasic potassium phosphate, 15 g of Bacto-agar (Difco), and trace elements (18). After autoclaving and cooling, the final pH of the medium was adjusted to pH 10.0 by the addition of an appropriate volume of sterile 20% (wt/vol) sodium carbonate. Strains were isolated on alkaline casein solid agar medium which contained (per liter) 10 g of casein, 1 g of dibasic potassium phosphate, 15 g of Bacto-agar, and trace element solution (18). The casein was solubilized by dissolution in 0.01 N NaOH prior to autoclaving. The pH of the casein medium was adjusted after autoclaving to 10.0 by addition of sterile sodium carbonate. Protease-producing strains were further characterized by cultivation on PSC solid agar medium containing 10% (vol/vol) skim milk.

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Sterile skim milk was added to the medium after autoclaving and cooling the medium to 50°C.

For fermentation of *Bacillus* sp. strain GX6638, PSC medium was modified to contain the following (grams per liter): potato starch, 10; Bacto-yeast extract, 5; Bacto-peptone, 5; dibasic potassium phosphate, 2; ammonium chloride, 1.0; and sodium thiosulfate pentahydrate, 2.5. After autoclaving, trace elements were added, and the pH was adjusted to 9.5 by addition of an appropriate volume of sterile sodium carbonate.

Strain isolation and characterization. *Bacillus* sp. strain GX6638 (ATCC 53278) was isolated by the following procedure. An alkaline soil sample (1 g) was suspended in 3.0 ml of distilled water by vigorous vortexing. A 0.1-ml portion of the soil suspension was plated directly onto alkaline casein solid agar medium and incubated at 37°C. After a 48-h incubation, individual colonies were picked and purified by streaking three times onto alkaline casein agar. Purified isolates were scored for extracellular protease production by streaking onto PSC agar medium containing skim milk. In situ protease production was demonstrated by the clearing of opaque milk proteins in the area surrounding isolated colonies growing on the surface.

For strain characterization, use was made of the work of Gordon et al. (8). Sporulation of *Bacillus* sp. strain GX6638 was induced by cultivation of cryogenically preserved cultures on PSC agar medium.

Fermentation. Fermentation of *Bacillus* sp. strain GX6638 was conducted in a 2-liter fermentor (model LH 500; LH Fermentation, Stoke Poges, England) with a working volume of 1.5 liters of modified PSC medium. Confluent overnight cultures of *Bacillus* sp. strain GX6638 on PSC agar were suspended in modified PSC medium and used as a source of inoculum. Fermentations were performed at 37°C, 800 rpm, and 1.0 volume of air per volume of medium per min and with no pH control.

Enzyme assays. For determining protease activity, the Delft method (A. G. van Velzen, British patent 1,353,317, May 1974), which measures the amount of trichloroacetic acid-soluble peptides from casein, was used, except that in most cases 0.4% (wt/vol) sodium tripolyphosphate (STPP) adjusted to pH 10.0 was used as a buffer. Thus, activities are expressed as alkaline Delft units (ADU). An ADU is an arbitrary unit which is defined as follows: if 1 ml of a 2% enzyme solution gives a ΔOD of 0.400, then the enzyme preparation has a protease activity of 1,000 ADU/g. All pH measurements represent final pH of reaction mixtures. The pHs were recorded with a Beckman pH meter equipped with a thermo-compensator; the pH meter was calibrated at the temperatures at which the reactions were performed.

Peptidase activity was determined by using succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (sAAPFpN) as the substrate as described by Del Mar et al.(6). The reaction mixtures contained, in a final volume of 0.7 ml, 0.5 mM sAAPFpN, 0.1 M CAPS [3-(cyclohexylamino)propanesulfonic acid] buffer (pH 10.5) and a suitable amount of enzyme. The increase in absorbance at 410 nm (ε = 8,480 M^{-1} cm^{-1}) due to the release of *p*-nitroaniline from the peptide was monitored spectrophotometrically. A unit is defined as the amount of enzyme required to form one micromole of product per minute.

Esterase activities were measured spectrophotometrically at 340 nm with *N*-carbobenzoxyl (CBZ)–glycine–*p*-nitrophenyl ester and CBZ–alanine–*p*-nitrophenyl ester as the substrates. Reaction mixtures (0.7 ml) contained 0.1 M Tris hydrochloride, pH 7.5, and 0.2 mM esterase substrate. An extinction coefficient of 5,500 M^{-1} cm^{-1} was used to calculate activities (4). A unit is defined as the amount of enzyme required to form one micromole of product per minute.

Protein concentration was determined as described by Lowry et al. (14).

Purification of proteases AP, AS, and HS. All purification procedures, unless otherwise stated, were performed between 0 and 5°C. Buffer A was 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid, potassium salt] buffer (pH 6.1 at 5°C), and buffer B was 50 mM Tris hydrochloride buffer (pH 8.0 at 5°C).

Crude culture broths were obtained by centrifugation of fermentation medium at 13,000 × *g* for 30 min. Total protease activity was precipitated by the addition of 80% (vol/vol) ice-cold acetone, followed by centrifugation. The protein pellet was suspended in a volume of buffer A equal to the original volume and brought to 80% saturation by addition of solid ammonium sulfate. The precipitated protein was collected by centrifugation, suspended in buffer A, and desalted on a Sephadex G-25 column equilibrated with buffer A. The eluate containing protease activity was applied to a quaternary ammonium-cellulose (QA52) column (2.5 by 7.5 cm) previously equilibrated with buffer A. The column was washed with three volumes of the same buffer, after which a continuous linear gradient from 0 to 0.4 M NaCl in a total volume of 600 ml of buffer A was applied; fractions of 10 ml were collected at a flow rate of 1 ml/min. Column fractions were monitored for protease activity as measured by the peptidase assay. Heat-stable protease activity was estimated by determining the residual peptidase activity of samples of column fractions that were incubated for 25 min at 60°C in 0.1 M CAPS buffer (pH 9.5) containing 5 mM EDTA.

Fractions of peak 1 containing protease AP and fractions of peak 2 containing proteases AS and HS were combined, and each pool was precipitated with 80% (vol/vol) cold acetone. Protein from peak 2 was collected by centrifugation, suspended in buffer B, and dialyzed against buffer B. The dialysate was applied to a column containing QAE Sephadex A-50 previously equilibrated in buffer B. The column was washed with this buffer, and protease AS and protease HS were resolved with a linear gradient of 0 to 0.8 M NaCl in buffer B; fractions were collected and monitored as above. Protease AS eluted between 0.2 and 0.25 M NaCl, whereas protease HS eluted between 0.26 and 0.32 M NaCl (data not shown).

Thermal inactivation kinetics. The thermal stability of the proteases was measured in 0.1 M CAPS (pH 9.4 or 10.5) containing either 5 mM EDTA or 2 mM calcium acetate or in 0.25% STPP, pH 11.5. The pH for each buffer were adjusted at the experimental temperature. Enzymes were added to buffer solutions preequilibrated at experimental temperatures, and samples were removed periodically and diluted into ice-cold buffer. Enzymatic activity remaining after treatment was determined from the initial velocity of peptide hydrolysis.

PAGE. Polyacrylamide gel electrophoresis (PAGE) of native proteins was done by the method of Laemmli (13), except sodium dodecyl sulfate (SDS) was omitted. Following electrophoresis in 8% polyacrylamide gels, proteins were electrophoretically transferred to nitrocellulose sheets (27), and proteases were identified by addition of peptide substrate directly to the nitrocellulose sheet. Protease activity was identified by the formation of discrete yellow bands that resulted from the hydrolysis of sAAPFpN. Experimental results were recorded by photographing the nitrocellulose

TABLE 1. Characteristics of *Bacillus* sp. strain GX6638

Cell morphology	
Vegetative cells	Motile rods, ends rounded, singular in general, but also occurring in pairs
Presporal forms	Extensive subterminal swelling prior to spore formation
Sporangia	Subterminal swelling
Spores	Ellipsoidal (0.6–0.8 by 1.2–1.4 μm)
Biochemical and cultural	
Growth temp (°C)	Maximum, 50–55, minimum, 10–15
Gram reaction	Positive
Nutrient agar (pH 7)	No growth
Nutrient agar (pH 7) plus 2% NaCl	Moderate growth
Nutrient agar (buffered at pH 9.5–10.5)	Excellent growth
Nutrient agar (pH 10) plus 7.5% NaCl	Growth
Pigment formation on glucose or tyrosine agar (pH 10)	Negative
Starch hydrolysis (pH 10)	Positive
Gelatin hydrolysis (pH 10)	Strong liquefaction
Casein hydrolysis (pH 10)	Positive
Lipid digestion on olive oil agar (pH 10)	Positive

sheets with the use of a Klett blue filter (400- to 450-nm band pass).

SDS-PAGE was done as described by Laemmli (13). Proteases were inactivated with PMSF prior to electrophoresis.

Isoelectric focusing. Analytical isoelectric focusing was performed in thin-layer polyacrylamide gels (Ampholine PAG plates) as specified by the manufacturer. After electrofocusing, proteases were visualized as described above. Alternatively, proteases were inactivated with PMSF prior to electrofocusing and stained with Coomassie brilliant blue R-250.

Immunodiffusion studies. Antiserum directed against subtilisin BPN' was prepared as described previously (Durham and Stewart, manuscript in preparation). Antiserum specific for bacillopeptidase F was kindly provided by J. Hageman. Immunodiffusion experiments were performed by the method of Ouchterlony (20); proteases were inactivated with PMSF prior to experiments.

Chemicals and reagents. All electrophoresis reagents were obtained from Bio-Rad Laboratories (Richmond, Calif.), electrofocusing gels, reagents, and standards were purchased from LKB Instruments (Rockville, Md.). The synthetic peptide sAAPFpN was from Calbiochem (San Diego, Calif.). QA52-cellulose and QAE-Sephadex were purchased from Whatman Ltd. (Maidstone, Kent, England) and Pharmacia Fine Chemicals, Inc. (Piscataway, N.J.), respectively. Hammersten casein was from United States Biochemical Corp. (Cleveland, Ohio), and low-molecular-weight SDS-PAGE standards and PMSF were obtained from Bethesda Research Laboratories (Gaithersburg, Md.). The following materials were purchased from Sigma Chemical Co. (St. Louis, Mo.): leupeptin, pepstatin, soybean trypsin inhibitor, trypsin inhibitor (chicken egg white type 11-0), iodoacetate, iodoacetamide, *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), EDTA, Trizma base, *p*-hydroxymercuribenzoate (HgBzOH), and subtilisin Carlsberg (type III). 3-Aminophenylboronic acid hemisulfate, *N*-dansyl-3-aminobenzeneboronic acid, and STPP were from Aldrich Chemical Co., Milwaukee, Wis. All other chemicals and reagents were the best grade commercially available.

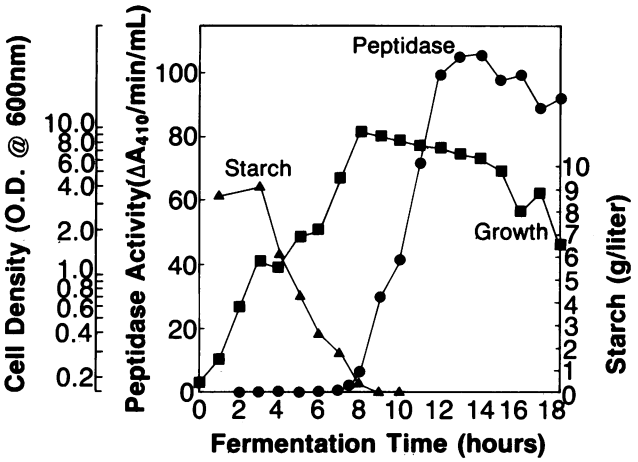

FIG. 1. Fermentation of *Bacillus* sp. strain GX6638. The fermentation was conducted in a 2-liter fermentor (1.5-liter working volume) as described in the text.

RESULTS

Characteristics of *Bacillus* sp. strain GX6638. The characteristics of isolate GX6638 are listed in Table 1. The isolate was an aerobic, sporeforming, motile, rod-shaped bacterium that belonged to the genus *Bacillus*. The sporangia were swollen with ellipsoidal subterminal spores.

Fermentation. The fermentation profile of *Bacillus* sp. strain GX6638 grown on modified PSC medium at pH 9.5 is shown in Fig. 1. Protease production was apparent after exogenous starch was exhausted, concurrent with stationary-phase growth and sporogenesis. The pH of the fermentation, although not controlled, did not fall below pH 8.8 (data not shown).

Resolution of multiple protease species. Preliminary heat inactivation experiments performed on crude culture broths obtained at intervals during the protease production phase of *Bacillus* sp. strain GX6638 were biphasic in nature (data not shown). The results suggested that more than one protease was produced by *Bacillus* sp. strain GX6638 during the stationary growth phase. The individual proteases were resolved by ion-exchange chromatography.

Two major peaks of protease activity were apparent following QA52-cellulose chromatography (Fig. 2). The first peak eluted between 0 and 0.1 M NaCl; electropherograms of fractions from the first peak indicated the presence of one protease species that exhibited mobility toward the anode in the buffer system used (Fig. 2B). This indicates that the protease, designated protease AP, had a basic isoelectric point (Fig. 2B). Protease AP (molecular weight, 22,000, and pI of 9.5) was extremely labile and was not investigated further.

A second peak of protease activity eluted between 0.15 and 0.23 M NaCl (Fig. 2). In the distal portion of this peak, heat-stable peptidase activity was detected, suggesting the presence of more than one protease species. This was further demonstrated by PAGE; the second activity peak was resolved into a minimum of two protease species. The heat-stable species, designated protease HS, exhibited rapid mobility during electrophoresis (Fig. 2C), whereas the major protease species, designated protease AS, was readily dif-

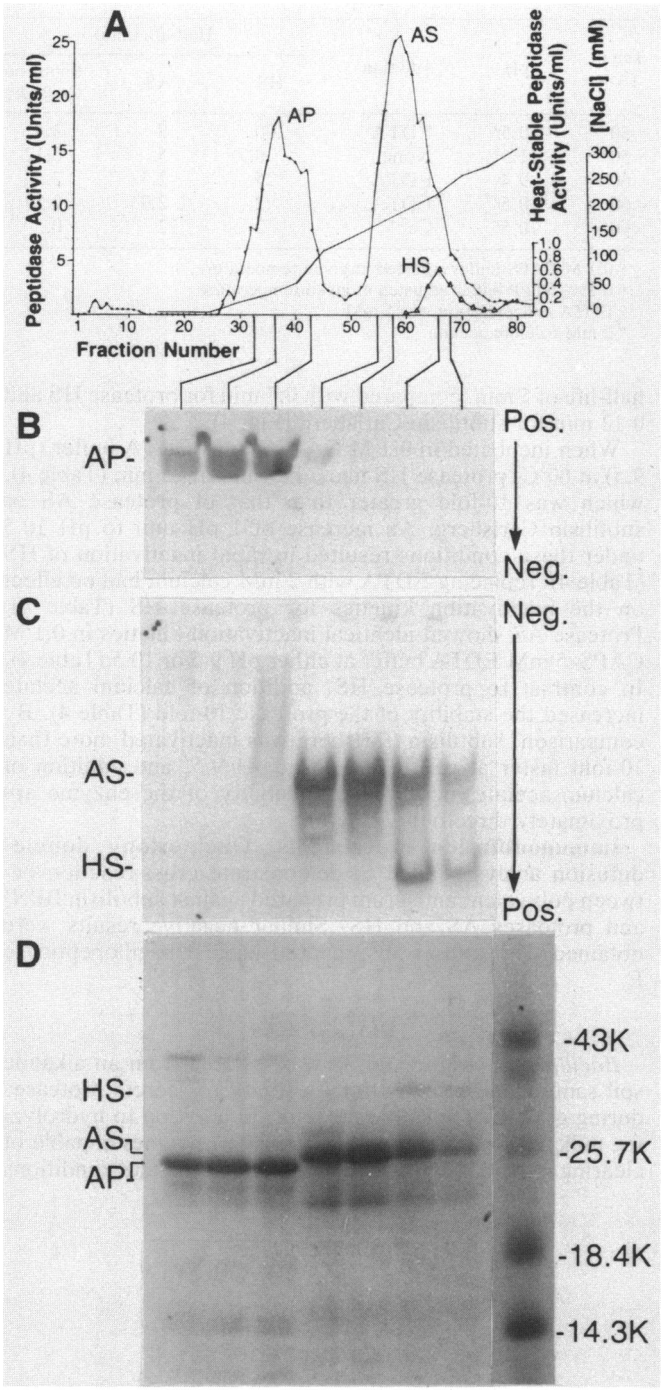

FIG. 2. Resolution of proteases from culture broths of *Bacillus* sp. strain GX6638. (A) QA52-cellulose chromatography of concentrated crude culture broths separated with a linear gradient of 0 to 0.4 M NaCl in 10 mM MES buffer, pH 6.1 (at 5°C). Fractions were assayed for peptidase activity (●) and heat-stable peptidase activity (▲) as described in the text. (B and C) PAGE of column fractions. Samples were electrophoresed as native proteins in 7% polyacrylamide gels (13) and electroblotted to nitrocellulose (27), and protease activities were identified by the hydrolysis of peptide substrate. The polarity of the gel shown in panel B is reversed from that of panel C to allow detection of protease species with basic isoelectric points. (D) SDS-PAGE of the same column fractions. Molecular weights (in thousands) are shown to the right.

TABLE 2. Specific activities of *Bacillus* sp. strain GX6638 proteases on various substrates[a]

Substrate	Sp act (U/mg of protein or ADU/mg of protein)		
	Protease AS	Protease HS	Subtilisin Carlsberg
Casein	3,803	1,944	5,237
sAAPFpNA	59	30	189
CBZ–Ala–*p*-nitrophenyl ester	83	295	59
CBZ–Gly–*p*-nitrophenyl ester	38	261	88

[a] Enzyme assays were performed as described in the text. Values with casein as the substrate are in ADU; other values are in units.

ferentiated from protease HS by its reduced electrophoretic mobility (Fig. 2C). In contrast to protease AP, the electrophoretic behavior of these enzymes was indicative of acidic proteins.

The mobilities of these proteins following SDS-PAGE of selected column fractions is shown in Fig. 2D. Protease HS and protease AS were further purified by QAE-Sephadex ion-exchange chromatography and were approximately 95% pure as judged by SDS-PAGE (not shown).

Properties of proteases AS and HS. The molecular weights of proteases AS and HS, as estimated by SDS-PAGE, were 27,500 and 36,000, respectively. Protease AS had an isoelectric point of 5.2, compared with ~4.2 for protease HS.

The activities of these enzymes with various substrates is shown in Table 2; subtilisin Carlsberg was included for comparison. Protease AS had twofold-higher proteolytic activity than protease HS, as demonstrated by hydrolysis of milk casein at pH 10 and 40°C. Similarly, the specific activity of protease AS with synthetic peptide substrate differed from that of protease HS by a factor of two. However, the K_{cat}/K_m ratio for this substrate was equivalent for protease AS (525 mM^{-1} s^{-1}) and protease HS (550 mM^{-1} s^{-1}). The specific activity of protease HS with CBZ–glycine–*p*-nitrophenyl ester was approximately sevenfold greater than that for protease AS.

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TABLE 3. Effect of inhibitors on protease activity[a]

Inhibitor	Concn	% of control activity	
		Protease AS	Protease HS
None (control)		100	100
PMSF	1 mM	0	0
EDTA	10 mM	114	100
Iodoacetate	10 mM	118	104
Iodoacetamide	10 mM	105	100
HgBzOH	1 mM	100	92
Leupeptin	0.1 mM	96	93
Pepstatin	0.01 mM	108	93
TLCK	1 mM	100	100
3-Aminophenylboronate	0.29 mM	46	71
N-Dansyl-benzeneboronate	0.014 mM	63	75
Trypsin inhibitor	0.1 mg/ml	36	97
	0.5 mg/ml	18	60
	1.0 mg/ml	9	31
Soybean trypsin inhibitor	0.1 mg/ml	93	103
	0.5 mg/ml	75	ND[b]
	1.0 mg/ml	57	97

[a] Proteases were incubated with inhibitors for 30 min at 25°C. Reactions were initiated by the addition of 0.5 mM sAAPFpNA.

[b] ND, Not determined.

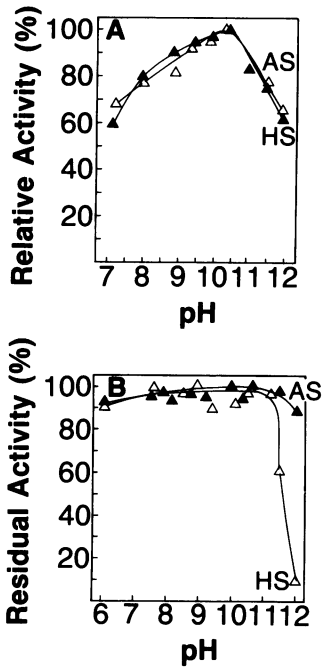

FIG. 3. pH optimum (A) and pH stability (B) of proteases AS (▲) and HS (△). The pH activity profiles were determined at 40°C in 0.4% STPP buffer as described in the text. pH stabilities were determined by measuring residual activity by the modified Delft method (pH 10) after incubation of proteases for 24 h at 25°C in 0.15% STPP adjusted from pH 6 to 12. pH values for both panels represent final pH determinations.

inhibitors on the activity of proteases AS and HS was determined by measuring residual peptidase activity following preincubation of enzymes with inhibitors in 50 mM Tris hydrochloride (pH 8.5) at 25°C (Table 3). The proteases were inhibited by an active-site inhibitor of serine proteases, PMSF, but not inhibitors of metallo- or thiol proteases. In general, inhibitor effects on both proteases were similar, except for the increased sensitivity of protease AS to trypsin inhibitors from egg white and soybean (Table 3).

Effect of pH on activity and stability. The pH optima for purified proteases AS and HS were determined at 40°C in 0.4% STPP buffer by a modified Delft method. Both proteases were active over a broad pH range, with maximal activities at pH 10.3 (Fig. 3A).

The stability of the enzymes as a function of pH is shown in Fig. 3B. Protease AS had exceptional alkaline stability; 88% of the initial activity was present after incubation at pH 12 for 24 h at 25°C. Protease HS had similar stability properties; no loss of activity was observed until pH 11.5 (60% retained).

Effect of temperature on activity and stability. The temperature optima for proteases AS and HS were determined at pH 8.5 and 10.0 in 0.4% STPP buffer by the Delft assay. Under these conditions, both proteases had a temperature optimum of 65°C at either pH (data not shown).

The thermal stability of protease AS and protease HS was measured at various temperatures and alkalinities and in the presence of EDTA or calcium acetate; subtilisin Carlsberg was examined for comparison (Table 4). At 50°C, proteases HS and AS had half-lives longer than 200 and 8 min, respectively, in 0.1 M CAPS (pH 10.5) containing 5 mM EDTA (Table 4). At pH 11.5, however, protease AS had a

TABLE 4. Summary of thermal inactivation kinetics

Temp (°C)	pH	Addition	Half-life (min)		
			HS	AS	Subtilisin Carlsberg
50	10.5[a]	EDTA[c]	>200	8	3.4
50	11.5[b]	None	0.7	5	0.12
60	9.4[a]	EDTA	25	2.3	2.5
60	10.5[a]	EDTA	2	2.3	0.17
60	10.5[a]	Ca^{2+}[d]	2	22	0.5

[a] 0.1 M CAPS buffer adjusted at given temperature.
[b] 0.25% STPP buffer adjusted at given temperature.
[c] EDTA concentration was 5 mM.
[d] 2 mM calcium acetate.

half-life of 5 min, compared with 0.7 min for protease HS and 0.12 min for subtilisin Carlsberg (Fig. 4).

When incubated in 0.1 M CAPS–5 mM EDTA buffer (pH 9.5) at 60°C, protease HS had a half-life of 25 min (Table 4), which was 10-fold greater than that of protease AS or subtilisin Carlsberg. An increase of 1 pH unit to pH 10.5 under these conditions resulted in rapid inactivation of HS (Table 4); replacing EDTA with 2 mM calcium had no effect on the inactivation kinetics for protease HS (Table 4). Protease AS showed identical inactivation kinetics in 0.1 M CAPS–5 mM EDTA buffer at either pH 9.5 or 10.5 (Table 4). In contrast to protease HS, addition of calcium acetate increased the stability of the protease 10-fold (Table 4). By comparison, subtilisin Carlsberg was inactivated more than 10-fold faster at pH 10.5 than at pH 9.5, and addition of calcium acetate increased the stability of the enzyme approximately threefold (Table 4).

Immunodiffusion experiments. Ouchterlony double-diffusion analysis failed to demonstrate cross-reaction between polyvalent antiserum prepared against subtilisin BPN' and proteases AS and HS. Similar negative results were obtained with antiserum prepared against bacillopeptidase F.

DISCUSSION

Bacillus sp. strain GX6638 was isolated from an alkaline soil sample and selected for its ability to secrete proteases during growth on alkaline medium. In addition to hydrolyzing milk casein, the bacterium secretes enzymes capable of clearing starch, gelatin, and lipids under alkaline conditions

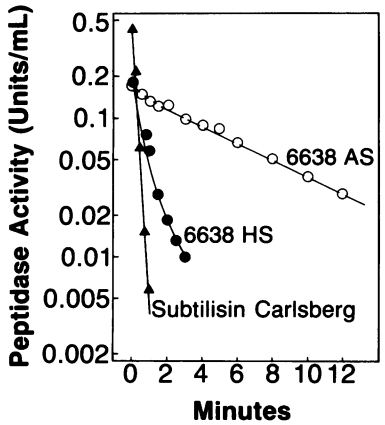

FIG. 4. Thermal inactivation kinetics of *Bacillus* sp. strain GX6638 proteases AS and HS and subtilisin Carlsberg in 0.25% STPP, pH 11.5.

TABLE 5. Comparison of proteases AS and HS with subtilisins Carlsberg and BPN'[a]

Enzyme	Mol wt	pI	pH optimum	Temp optimum (°C)	Heat stability (min)[b]	Alkali stability (%)[c]	Ca^{2+} stabilization[d]
Proteases							
AS	27,500	5.2	10.3	65	8.0	100	+
HS	36,000	4.2	10.3	65	>200	60	−
Subtilisins							
Carlsberg	27,300	9.4	10.5	60	3.4	20	+
BPN'	27,500	7.8	10.5	60	2.4	1	+

[a] Subtilisins Carlsberg and BPN' (16, 19) were chosen as representatives of group A and B subtilisins, respectively, as proposed by Keay et al. (12).
[b] Half-life of enzymatic activity after incubation at 50°C in 0.1 M CAPS (pH 10.5)–5 mM EDTA.
[c] Percent of activity remaining after 24 h of incubation at pH 11.5 and 25°C.
[d] Ability of Ca^{2+} to stabilize activity.

(Table 1). The classification of *Bacillus* sp. strain GX6638 is unclear. It grew preferentially on alkaline medium, but grew at pH 7 if the medium was supplemented with NaCl (Table 1). At present there are no direct classification schemes for determining the species of alkalophilic *Bacillus* strains. Gordon and Hyde (9) have classified numerous alkalophilic bacilli into the *Bacillus firmis-Bacillus lentus* complex after isolating variants adapted to growth at pH 7. Whether such variants are altered in other physiological properties is not known.

The results of the present investigation demonstrate that *Bacillus* sp. strain GX6638 produces a minimum of three distinct proteases at the end of the logarithmic growth phase (Fig. 2). To our knowledge this is the first example of an alkalophilic *Bacillus* sp. that produces multiple proteolytic enzymes. Of even greater significance is the observation that all three proteolytic enzymes characterized in this study could be classified as alkaline proteases. In general, *Bacillus* species that produce multiple proteases synthesize neutral as well as alkaline proteases (15, 22, 23). In this regard, *Bacillus* sp. strain GX6638 appears to be unique. Moreover, we have demonstrated that two of the three alkaline proteases produced by *Bacillus* sp. strain GX6638 were relatively atypical compared with previously identified alkaline proteases (Table 5). The typical alkaline protease or subtilisin described to date is a low-molecular-weight protein with a high isoelectric point that is inactivated by serine active-site reagents, but not sulfhydryl or metal chelating agents (16, 19) (Table 5). Although proteases HS and AS share several of these properties, we have demonstrated that these two proteases are acidic proteins that exhibit unusual alkaline and thermal stability characteristics compared with proteases from other alkalophilic *Bacillus* spp. (2, 11) and subtilisins from neutralophilic bacilli (16, 19) (Table 5).

Proteases AS and HS hydrolyzed casein over a broad pH range (Fig. 3) and were most active at 65°C. However, protease AS had higher proteolytic activity with casein as the substrate (Table 2). Furthermore, it had a lower molecular weight and differed from protease HS in sensitivity to trypsin inhibitors (Table 3) and stability properties (Table 4); protease HS showed higher esterolytic activity than protease AS (Table 2). From these data, we conclude that proteases AS and HS represent distinct protease species and that protease HS is not an unprocessed form of protease AS. Evidence to the contrary must await primary sequence comparisons.

Protease HS had physical and catalytic properties similar to those of bacillopeptidase F (3, 24) and an intracellular protease (ISP-1) (25, 26) from *Bacillus subtilis*. These serine proteases all exhibit rapid electrophoretic mobilities in basic buffer systems, possess low isoelectric points (~4.2 to 4.4), similar molecular weights (31,000 to 36,000), and high esterolytic and low proteolytic activity (3, 24–26). In addition, these alkaline proteases are serologically remote from subtilisins (10; this study). However, several properties indicate that protease HS is distinct from these proteases; ISP-1 differs from protease HS in its dependence on Ca^{2+} for stability, its negligible proteolytic activity, and its temperature optimum of 40°C (25, 26). Moreover, polyvalent antiserum directed against bacillopeptidase F failed to cross-react with protease HS. Protease HS may represent a homolog of bacillopeptidase F or ISP-1 of alkalophilic *Bacillus* origin. Definitive proof of whether these proteins evolved from a common ancestral gene must await primary amino acid sequence comparisons.

The most interesting characteristics of proteases HS and AS were their stability properties. Protease AS was extremely alkaline stable (Fig. 3). The protease retained almost complete activity after a 24-h incubation at pH 12. Under these conditions, but at pH 11.5, subtilisin Carlsberg and subtilisin BPN' retained 20 and 1%, respectively, of their initial proteolytic activity (Table 5). The pH had little effect on the half-life of the protease during incubation at elevated temperatures (Table 4), and the addition of Ca^{2+} was shown to stablize the enzyme by increasing the half-life 10-fold at pH 10.5 and 60°C. Protease HS was inherently more resistant to thermal inactivation. In the presence of EDTA, protease HS had a half-life of more than 200 min at pH 10.5 and 50°C. By comparison, under these conditions subtilisin Carlsberg had a half-life of 3.4 min and subtilisin BPN' a half-life of 2.4 min (Table 5). Unlike with protease AS, increasing the pH to 11.5 had a dramatic effect on enzyme half-life at 50°C (Table 4). This was also observed at 60°C; a pH increase of 1 unit (pH 9.4 to pH 10.5) resulted in a 10-fold reduction in half-life (Table 4). Addition of Ca^{2+} had no effect on the inactivation kinetics of protease HS, suggesting that the protein is not stabilized by Ca^{2+}. This is in contrast to most alkaline proteases, which are stabilized significantly by Ca^{2+} (11, 12, 16, 23) (Table 5).

Proteolytic enzymes from *Bacillus* spp. are presently used as detergent supplements for removal of proteinaceous stains (1, 2, 16). However, one disadvantage of using these proteases is their reduced shelf-life under highly alkaline conditions. Currently, alterations in the structural gene encoding subtilisin BPN' via site-directed mutagenesis has resulted in enzymes with better stability properties (5, 7, 21). For example, the introduction of disulfide linkages in subtilisin BPN' has produced a variant protein with a melting temperature 3.1°C higher than that of the wild-type protein and a two-fold increase in half-life at 45°C (21). Furthermore, random site-directed mutagenesis of subtilisin BPN', resulting in a modification of the hydrogen-bonding properties, produced a mutant enzyme with enhanced thermal (5) as well as alkaline (unpublished data) stability characteristics. An advantage of random site-directed mutagenesis is that with a rapid screening procedure, mutants with enhanced stability properties can be derived at a frequency of less than 1 per 1,000 screened (5). At present, however, predicting specific amino acid changes that affect the desired functions(s) is difficult. In this regard, a comparative study of the structures of proteases HS and AS with other alkaline proteases may provide insight into the criteria governing the alkaline as well as thermal stabilities of proteins. A direct consequence of such a study could be the construction of

enhanced-stability variants of highly active proteolytic enzymes, such as subtilisins Carlsberg (1, 16, 19), BPN′ (16, 19), and GX (Durham and Stewart, submitted for publication), for industrial applications.

In summary, the available evidence suggests that proteases HS and AS represent novel serine proteases from the genus *Bacillus*. They differ from other alkaline proteases from alkalophilic bacilli (2, 4) and from the subtilisins (12, 16, 19) (Table 5) in their acidic isoelectric points and their unique stability properties. Our data indicate that protease AS is unusually alkaline stable, whereas protease HS is a heat-stable protease, unique in that it does not appear to require Ca^{2+} for stability.

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