Appl Microbiol Biotechnol (1990) 34:57-62

Applied and Microbiology Biotechnology

Alkaline serine protease produced from citric acid by *Bacillus alcalophilus* subsp. *halodurans* KP 1239

Yukio Takii, Naohiro Kuriyama, and Yuzuru Suzuki

Department of Agricultural Chemistry, Kyoto Prefectural University, Shimogamo, Sakyo-ku, Kyoto 606, Japan

Received 2 February 1990/Accepted 11 May 1990

Summary. Maximum production of alkaline serine protease by *Bacillus alcalophilus* subsp. *halodurans* KP 1239 was achieved after 24 h cultivation, at an initial pH of 7.6, on a medium containing 1.0% sodium citrate, 0.3% yeast extract, and 0.3% KH_2PO_4. The enzyme was purified to crystalline form from culture broth. The enzyme was most active at 60° C and at pH 11.5. The molecular weight, isoelectric point and sedimentation coefficient in water at 20° C were estimated as 29000, 8.8 and 3.3*S*, respectively. The N-terminal amino acid sequence was Ala-Gln-Ser-Val-Pro-Trp-Gly-Ile-Ser-Arg-Val-Gln-Ala-Pro-Ala-Ala-His-Asn-Arg-Gly-. The enzyme shared its antigenic determinants with *B. alcalophilus* ATCC 21522 serine protease, but not with the subtilisins Carlsberg and BPN′.

Introduction

Serine alkaline protease produced by alkalophilic *Bacillus* strains are much more active and stable at considerably higher pH than the subtilisins Carlsberg and BPN′ (EC 3.4.21.14) (Ward 1983). Complex media with high alkalinity are used for eminent protease production by these bacilli (Aunstrup et al. 1972; Horikoshi and Akiba 1982; Tsuchida et al. 1986; Fujiwara and Yamamoto 1987; Takami et al. 1989). In 1982, we isolated a strain (KP 1239) of *B. alcalophilus* subsp. *halodurans*, which produced a serine protease able to function most actively at pH 11.5. In the present study, the enzyme production by strain KP 1239 and characterization of the enzyme are reported.

Materials and methods

Screening. Soil samples (each 0.1 g) collected around this University and in the vicinities of Asahi (Osaka) and Shimogamo (Kyoto) were added to test tubes (1.8 × 18 cm), each containing 5 ml medium I [0.3% (w/v) KH_2PO_4, 0.2% yeast extract, 1.0% casein, 0.2% peptone, 0.002% $MgSO_4 \cdot 7H_2O$, 0.005% $FeSO_4 \cdot 7H_2O$, 0.0002% $MnCl_2 \cdot 4H_2O$, 0.0001% $Na_2MoO_4 \cdot 2H_2O$, 0.002% $CaCl_2 \cdot 2H_2O$ and 1% Na_2CO_3 (pH 10.3 after autoclaving; Boyer et al. 1973)], and incubated for 48 h at 37° C with the tubes leaning at an angle of about 10°. After one more enrichment of cultivation on medium I, 120 cultures were obtained by streaking the samples on medium I agar (3%) plates followed by incubation for 18 h at 37° C. Cells of each isolate, grown for 18 h at 37° C on a medium I agar slant (8 ml/tube), were suspended in 5 ml of 0.85% NaCl, inoculated to an absorbance at 660 nm (A_{660}) of 0.02–0.03 in a test tube containing 5 ml medium I, and shaken at 37° C for 8 h at 110 oscillations/min (6.5-cm amplitude) on a reciprocal shaker. The culture was centrifuged (6° C, 30 min, 8000 *g*), and the supernatant was assayed for protease. Of 120 cultures, 11 with protease activity were streaked again on medium I plates. Among 132 colonies selected, protease production exceeded 3.11 units (U)/ml culture with 43 strains that included KP 1239, KP 1241, KP 1242 and KP 1243 producing 3.48, 3.11, 3.70 and 3.59 U/ml, respectively. These four strains all were derived from soils around this University.

Strain KP 1239. Strain KP 1239, which grew at 24°–53° C and best at 45° C on medium II [0.3% KH_2PO_4, 0.5% $NaHCO_3$, 0.5% peptone and 0.5% yeast extract (pH 8.3)], was assigned to a strain of *B. alcalophilus* subsp. *halodurans* (Boyer et al. 1973; Claus and Berkeley 1986). The motile, amylolytic, proteolytic, rod-shaped vegetative cells (0.9–1.2 × 10–15 μm) were positive to Gram, catalase, and oxidase reactions, and produced terminally swollen sporangia with oval spores (0.9–1.2 × 1.2–1.3 μm) after 18 h growth at 37° C on medium II. The initial growth pH range was from 6.5 to 10.1 (optimum at pH 8.8) on medium II. The microbe grew on medium II with 12% NaCl, but not with 15% NaCl or 0.02% NaN_3. The DNA (Miura 1967) showed 42.0 mol% of guanine/cytosine (GC) content (Marmur and Doty 1962). The following properties were also established: anaerobic growth on medium II containing 0.1% glucose; negative urease reaction; NO_3^- reduction to NO_2^- without N_2 evolution; negative Voges-Proskauer reaction; negative methyl red test; no indole formation; no reduction of methylene blue; acid production from glucose, fructose, sucrose, mannitol, arabinose, salicin, sorbitol, lactose, glycerol, maltose and soluble starch; no acid formation from xylose; no gas produced from the above sugars; citrate assimilation on Christensen medium supplemented with 0.45 *M* KCl. All the properties presented above were identical with those of strains KP 1241, KP 1242 and KP 1243.

Enzyme assay. The mixture (2.5 ml) containing 44 m*M* borate-NaOH (pH 10.0), 0.5% casein and enzyme (0.5 ml, 0.1–0.3 U) was

Offprint requests to: Yuzuru Suzuki

incubated at 37° C for 2–10 min, mixed with 1 ml of 1.5 *M* trichloroacetic acid/0.525 *M* acetic acid/0.35 *M* sodium acetate, and heated at 60° C for 10 min prior to centrifugation. The A_{275} (1-cm light path) of the supernatant was measured and converted to the amount of tyrosine equivalent (Takii et al. 1987). One unit (U) of enzyme activity was defined as the amount of enzyme releasing 1 μmol tyrosine equivalent/min. Protein was assessed by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Enzyme purification. Cells of *B. alcalophilus* subsp. *halodurans* KP 1239, grown at 37° C for 18 h on an agar slant of medium III [0.1% $(NH_4)_2SO_4$, 0.1% yeast extract, 0.02% KCl and 0.02% $MgSO_4 \cdot 7H_2O$ (pH 8.5)], were suspended in 5 ml of 0.85% NaCl, and 0.1 ml suspension was transferred into a 500-ml Sakaguchi flask containing 80 ml medium III. The flask was shaken for 18 h at 37° C at 110 oscillations/min (6.5-cm amplitude). The 4-ml cultures were inoculated into 12 flasks, each containing 80 ml of 1% sodium citrate, 0.3% KH_2PO_4 and 0.3% yeast extract, pH 7.6. Shaking cultivation was continued for 28 h. All subsequent operations were carried out at 4°–6° C. The cultures were centrifuged. The pH of the supernatant (915 ml; 7050 U; 7.8 U/mg protein) was adjusted to 7.5 with 5 *M* KH_2PO_4. The solution was mixed by stirring for 30 min with 500 ml of diethylaminoethylcellulose equilibrated with 10 m*M* TRIS-HCl/2 m*M* $CaCl_2$ (pH 7.5; buffer A), and filtered on Toyo (Tokyo, Japan) filter paper No. 1. The cellulose was washed with 200 ml buffer A. The washing and the filtrate were combined (1240 ml; 7440 U; 12.5 U/mg protein), and dialysed against 10 l buffer A (four buffer changes for 12 h each). The dialysate was mixed for 2 h with 500 ml carboxymethylcellulose equilibrated with buffer A, and filtered through the filter paper. The cellulose was washed twice with 500 ml of buffer A and eluted with every 100 ml of 0.1 *M* glycine-NaOH/2 m*M* $CaCl_2$ (pH 11.0) at a rate of 2.0 ml/min. The active fractions eluted between 100 ml and 500 ml of this buffer (485 ml; 6940 U; 33.7 U/mg protein) were combined, concentrated to 24 ml by ultrafiltration on an Amicon (Danvers, MA, USA) PM-10 filter, and saturated to 60% with solid $(NH_4)_2SO_4$. The resulting precipitate was recovered by centrifugation, washed with 60% $(NH_4)_2SO_4$ and diluted twofold with 20 m*M* TRIS-HCl/2 m*M* $CaCl_2$ (pH 8.8, buffer B). Rod-like crystals appeared within 1 h and crystallization was completed by standing for 3 h.

The crystals recovered by centrifugation were washed with buffer B/30% $(NH_4)_2SO_4$ and dissolved in 2 ml of 0.2 *M* NaOH before centrifugation (4410 U; 33.9 U/mg protein). The pH of the supernatant was adjusted to 8.8 by dropwise addition of 0.2 *M* CH_3COOH, and the volume was increased to 4 ml with buffer B. The solution was brought to 30% $(NH_4)_2SO_4$ saturation and kept overnight for crystallization (Fig. 1). This procedure was repeated. The third crystals were suspended in 650 μl of buffer B (928 U; 13.2% yield; 31.3 U/mg protein) and stored at −30° C. The *B. alcalophilus* ATCC 21522 alkaline protease was purified to homogeneity (crystalline form) (425 U; 9.7% yield; 30.2 U/mg protein), as described by Horikoshi (1971).

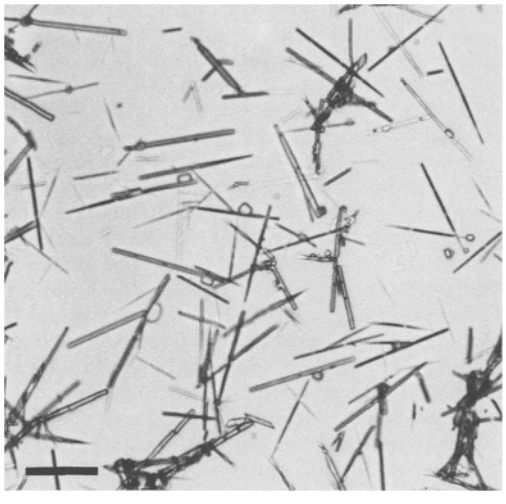

Fig. 1. Crystals of alkaline protease produced by *Bacillus alcalophilus* subsp. *halodurans* KP 1239. The micrograph of crystals was taken with an Olympus PM-10-M camera attached to an Olympus FHT-533 microscope. Magnification ×50. The *bar* indicates 600 μm

Test tube cultivation. Cells of *B. alcalophilus* subsp. *halodurans* KP 1239 grown at 37° C for 18 h on a medium III slant were suspended in 5 ml of 0.85% NaCl. The 0.1 ml suspensions were added to test tubes each containing 5 ml medium, shaken at 37° C for 24 h at 110 oscillations/min, and centrifuged. The supernatant was assayed for final pH and protease activity. Cell yield was estimated turbidimetrically and expressed as dry cell weight (Suzuki et al. 1976).

Results

When cultivated on a medium (initial pH 7.0) containing 0.3% KH_2PO_4, 0.3% yeast extract, 0.45 *M* KCl and one of various carbon or nitrogen sources (1%), *B. alcalophilus* subsp. *halodurans* KP 1239 effectively produced protease from citrate, fumarate, succinate, soluble starch, casein, peptone or casamino acid (Table 1).

Table 1. Effects of various carbon and nitrogen sources on the production of alkaline protease by *Bacillus alcalophilus* subsp. *halodurans* KP 1239[a]

Additions (1%)	Protease (units [U]/ml)	Cell yield (mg/ml)	Specific protease yield (U/mg of cells)	Final pH
None	0.01	0.65	0.02	8.4
Citrate[b]	7.03	1.50	4.69	9.0
Citric acid	7.10	1.51	4.70	9.0
Fumaric acid	5.60	1.50	3.73	9.2
Succinic acid	3.90	1.51	2.60	9.8
Malic acid	2.07	1.80	1.15	9.0
Acetic acid	0.68	2.16	0.31	9.6
Glucose	0.00	0.38	0.00	5.3
Glycerol	0.03	0.68	0.04	5.7
Fructose	0.00	0.08	0.00	6.3
Lactose	0.08	1.59	0.05	7.3
Soluble starch	9.11	2.02	4.51	7.2
Casein	5.10	2.42	2.13	8.8
Peptone	7.89	1.93	4.09	8.8
Casamino acid	6.89	1.90	3.96	8.9
Urea	0.16	0.24	0.67	8.1
$NaNO_3$	1.42	1.13	1.26	8.4
NH_4NO_3	0.10	1.18	0.08	7.3
NH_4Cl	0.26	1.16	0.22	7.2
$(NH_4)_2SO_4$	0.25	1.14	0.22	7.2

[a] The medium contained 0.3% KH_2PO_4, 0.3% yeast extract, 0.45 *M* KCl and one of the compounds (1%) presented above. The pH was adjusted to 7.0 with 1 *M* NaOH or HCl before autoclaving. Cultivation was for 24 h at 37° C as described in Test tube cultivation, Materials and methods

[b] Trisodium salt

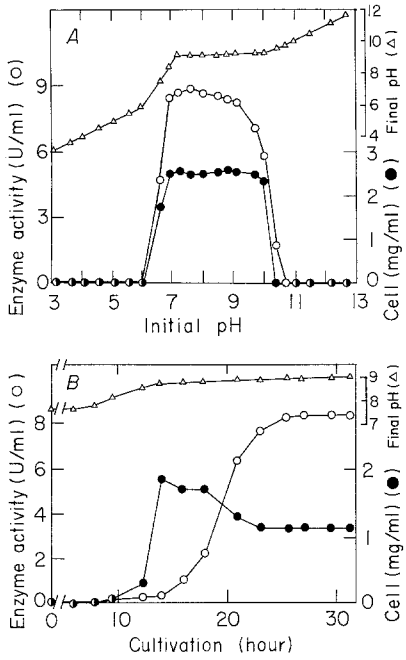

Fig. 2. A Effect of initial culture pH on growth (-●-) of *B. alcalophilus* subsp. *halodurans* KP 1239 on alkaline protease production (-○-) and on final culture pH (-△-). Medium (5 ml/tube) contained 1% sodium citrate, 0.3% yeast extract and 0.3% KH_2PO_4. The pH was varied with 1 *M* HCl, 1 *M* NaOH (pH 6.0–8.6), 10% Na_2CO_3 (pH 9.0–10.8) or Na_2CO_3/10% NaOH (pH 11.5–13.0). Cultivation was at 37° C for 24 h, as in Test tube cultivation, Materials and methods. **B** Time course of alkaline protease production (-○-) and growth (-●-), and change in culture pH (-△-). Shaking cultivation was at 37° C with 500-ml Sakaguchi flasks each containing 80 ml of the same medium (pH 7.6), as in Enzyme purification, Materials and methods. Protease activity (units [U]/ml of culture), cell concentration (dry cell weight, mg/ml) and culture pH were determined as in Materials and methods

Of these, citrate gave the highest specific yield (4.7 U/mg of dry cells), which was about 240-fold more than the yield without citrate. The protease production was optimal between initial pH 6.9–9.1 with the highest at pH 7.6 on a medium containing 1% sodium citrate, 0.3% KH_2PO_4 and 0.3% yeast extract (Fig. 2A). The maximum enzyme production was achieved after 24 h cultivation (about 8 U/ml of culture) on the same medium with an initial pH of 7.6 (Fig. 2B).

The final crystalline preparation of *B. alcalophilus* subsp. *halodurans* KP 1239 protease was purified four-fold over culture broth, the yield being about 13%. The sample gave a single protein band on sodium dodecyl (SDS)-polyacrylamide gel electrophoresis (Weber and Osborn 1969) (Fig. 3). The molecular weight (mol. wt.), sedimentation coefficient at 20° C in water ($s_{20,w}$) and isoelectric point (pI) were estimated as 29000, 3.3*S* and 8.8, respectively (Weber and Osborn 1969; Martin and Ames 1961; Wrigley 1968). The amino acid composition and the N-terminal sequences of 20 residues are shown in Table 2 and Fig. 4, respectively.

Rabbit antiserum against *B. alcalophilus* ATCC 21522 protease produced single precipitin lines on Ouchterlony double immunodiffusion with this enzyme and proteases produced by strains KP 1239, KP 1241, KP 1242 and KP 1243 of *B. alcalophilus* subsp. *halodurans* (Fig. 5, plate a). The lines completely fused without a spur. The antiserum failed to react on the subtilisins Carlsberg and BPN′ (Fig. 5, plate b).

B. alcalophilus subsp. *halodurans* KP 1239 protease was most active at 60° C or at 70° C, respectively, in the

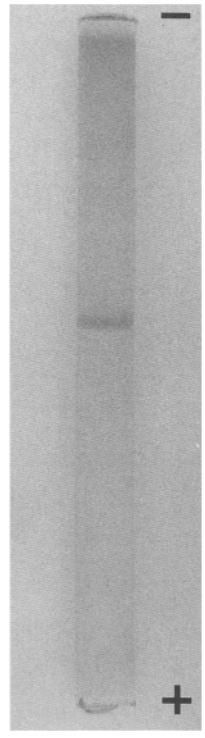

Fig. 3. Sodium dodecyl sulphate (SDS)-gel electrophoresis of purified alkaline protease of *B. alcalophilus* subsp. *halodurans* KP 1239. The enzyme (10 µg in 5 µl H_2O) was mixed with 5 µl of 2 m*M* phenylmethylsulphonylfluoride (PMSF), incubated for 30 min at 4° C and treated with 2 µl of 2 *M* formic acid before incubation for 1 h at 22° C. The solution was mixed with 20 µl of 10% SDS, boiled for 5 min and subjected to SDS-electrophoresis for 8 h at 5 mA/tube at 25° C (Weber and Osborn 1969). The gel (diameter = 0.5 cm) was stained with Coomassie brilliant blue (Weber and Osborn 1969)

Table 2. Amino acid composition of alkaline proteases from *B. alcalophilus* subsp. *halodurans* KP 1239 (I), *B. alcalophilus* ATCC 21522 (II) and *Bacillus* sp. 221 (III) and of the subtilisins Carlsberg (IV) and BPN′ (V)[a]

	I[b]	II[b]	III[c]	IV[d]	V[d]
Aspartic acid	9.8	11.5	10.1	10.2	10.2
Threonine	6.8	6.9	6.3	6.9	4.7
Serine	11.7	11.6	8.0	11.7	13.5
Glutamic acid	6.1	5.8	5.6	4.4	5.5
Proline	5.0	3.6	5.6	3.3	5.1
Glycine	12.6	13.7	13.6	12.8	12.0
Alanine	15.2	16.5	15.6	15.0	13.5
Cysteine	0.0	0.0	0.0	0.0	0.0
Valine	10.5	10.1	9.4	11.3	10.9
Methionine	1.3	1.2	1.3	1.8	1.8
Isoleucine	2.8	2.7	3.1	3.6	4.7
Leucine	7.3	6.0	7.7	5.8	5.5
Tyrosine	2.8	2.4	3.1	4.7	3.6
Phenylalanine	0.68	0.84	0.70	1.5	1.1
Lysine	2.4	2.6	2.0	3.3	4.0
Histidine	2.4	2.6	2.8	1.8	2.2
Arginine	2.7	2.8	2.8	1.5	0.73
Tryptophane	N[e]	N[e]	1.7	0.36	1.1

[a] Data expressed as mol% of residues
[b] Determined after hydrolysis (1 mg protein used) in 6 *M* HCl for 24 h at 110° C (Suzuki and Tomura 1986)
[c] From Horikoshi (1971)
[d] From Delange and Smith (1968)
[e] N = not determined

```
     1               5                  10                  15                  20
I.   Ala-Gln-Ser-Val-Pro-Trp-Gly-Ile-Ser-Arg-Val-Gln-Ala-Pro-Ala-Ala-His-Asn-Arg-Gly-
II.  Ala-Gln-Ser-Val-Pro-Trp-Gly-Ile-Ser-Arg-Val-Gln-Ala-Pro-Ala-Ala-His-Asn-Arg-Gly-
III. Ala-Gln-Thr-Val-Pro-Tyr-Gly-Ile-Pro-Leu-Ile-Lys-Ala-Asp-Lys-Val-Gln-Ala-Gln-Gly-
IV.  Ala-Gln-Ser-Val-Pro-Tyr-Gly-Val-Ser-Gln-Ile-Lys-Ala-Pro-Ala-Leu-His-Ser-Gln-Gly-
```

Fig. 4. N-Terminal sequences of alkaline proteases from *B. alcalophilus* subsp. *halodurans* KP 1239 (*I*) and *B. alcalophilus* ATCC 21522 (*II*) and of the subtilisins Carlsberg (*III*) and BPN′ (*IV*) (Smith et al. 1968). KP 1239 and ATCC 21522 proteases (each 1.7 nmol) were subjected to automated Edman degradation, followed by analysis of phenylthiohydantoin amino acids on an Applied Biosystems (Foster City, CA, USA) model 120A PTH analyser

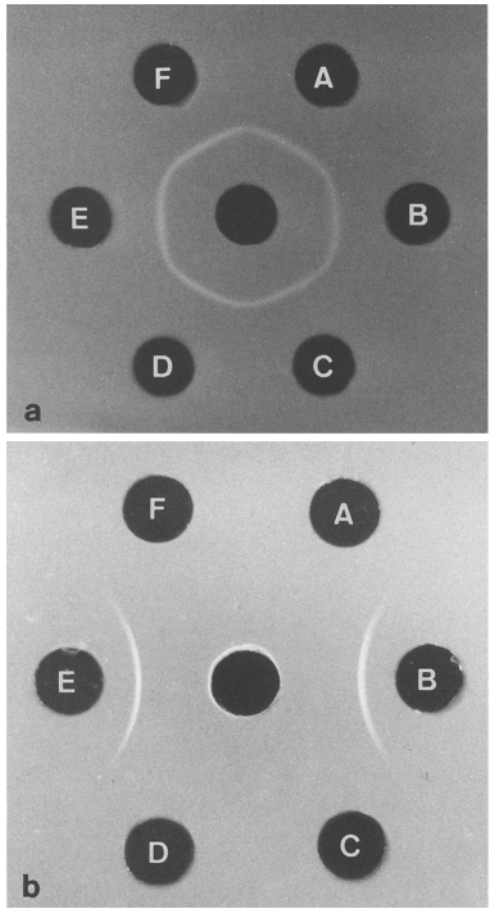

Fig. 5. Double immunodiffusion. Rabbit antiserum (6 µl) (Suzuki et al. 1980) prepared against *B. alcalophilus* ATCC 21522 alkaline protease was added to the centre wells (3 mm diameter) in plates *a* and *b* each consisting of 1.5% agarose and 25 m*M* borate/0.1% NaN_3/1 m*M* PMSF. Wells *B* and *E* of plates *1* and *2* contained ATCC 21522 protease (10 µl, 1.1 U). Wells *A* and *D* of plate *1* contained *B. alcalophilus* subsp. *halodurans* KP 1239 alkaline protease (10 µl, 1.3 U); wells *C* and *F* contained one of the culture supernatants (each 1.0 U protease) of *B. alcalophilus* subsp. *halodurans* KP 1241, KP 1242 and KP 1243. Wells *A* and *D* of plate *2* contained subtilisin BPN′ (1.1 U, 90 µg); wells *C* and *F* contained subtilisin Carlsberg (1.3 U, 102 µg). Each enzyme sample or culture supernatant used was treated at 4° C for 1 h with 1 m*M* PMSF before application on the plates. Immunodiffusion on plates *1* and *2* was carried out at 4° C for 28 h. The plates were soaked in 0.85% NaCl/0.1% NaN_3 at 22° C overnight and photographed (Suzuki et al. 1980). One microlitre of the antiserum completely precipitated 0.12 U of ATCC 21522 enzyme and 0.12 U of KP 1239 enzyme, respectively

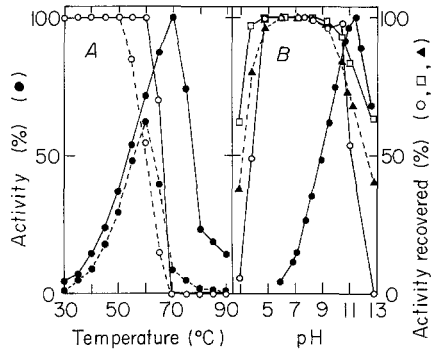

Fig. 6. **A** Effect of temperature on the activity (●) and stability (○) of *B. alcalophilus* subsp. *halodurans* alkaline protease. The activity was determined as in Enzyme assay, Materials and methods, except that the incubation temperature was changed in the presence (-●-) or absence (--●--) of 5 m*M* $CaCl_2$ [apparent activation energies: 15 kJ/mol (40°-60° C) or 12 kJ/mol (40°-70° C), respectively]. The enzyme (0.26 U/ml) was incubated for 10 min at different temperatures in 10 m*M* TRIS-HCl (pH 7.5) (--○--) or in 10 m*M* TRIS-HCl (pH 7.5)/5 m*M* $CaCl_2$ (-○-). The mixture was assayed for remaining activity. **B** Effect of pH on the activity (-●-) and its stability (-○-, -□-, --▲--). The activity was assayed as in Enzyme assay, Materials and methods, except that the pH of reaction mixture was altered by using 44 m*M* sodium acetate-HCl (pH 3.0-6.5), 44 m*M* TRIS-HCl (pH 6.6-8.3) or 44 m*M* glycine-NaOH (pH 9.0-13.0). The enzyme (1.09 U/ml) in 10 m*M* borate-NaOH (pH 10.0) was mixed with 10 vol of the above buffers (0.1 *M*), incubated at 4° C (-□-) or 25° C (--▲--) for 24 h, or at 55° C (-○-) for 10 min. The mixture was assayed for remaining activity after tenfold dilution with 10 m*M* borate-NaOH (pH 10)

Table 3. The K_m and V values of *B. alcalophilus* subsp. *halodurans* KP 1239 protease for protein substrates[a]

Substrate	K_m (mg/ml)	V (µmol tyrosine equivalent formed/ min/mg enzyme)
Casein	0.4	47.0
α-Casein	0.6	28.0
κ-Casein	0.4	25.0
Bovine serum albumin	2.9	3.3
Haemoglobin	3.4	3.7

[a] The hydrolysis of protein substrate was assayed as in Enzyme assay, Materials and methods, except that 0.13-0.15 U enzyme was used and the substrate level was varied between 0.75 and 5.0 mg protein/ml. The K_m and V were determined by the method of Lineweaver and Burk (1934)

absence or presence of 5 m*M* Ca^{2+} (Fig. 6). The optimum pH for activity was 11.5 (Fig. 6). The stability against heat and pH is depicted in Fig. 6.

The Michaelis constant (K_m) and maximum initial velocity (V) of hydrolysis for protein substrates are compared in Table 3. The enzyme activity was inhibited 88% by 1 m*M* diisopropylfluorophosphate, 89% by 10 m*M* phenylmethylsulphonylfluoride (PMSF), 75% by 6.9 m*M* SDS and 40% by 40% ethanol, but not at all by 1 m*M* ethylenediaminetetraacetate, which was tested as in Enzyme assay, Materials and methods, except that the reaction mixture contained one of these inhibitors.

Discussion

Despite of their many common phenotypic properties, *B. alcalophilus* subsp. *halodurans* can be definitely distinguished from *B. alcalophilus* by its abilities to grow at far higher temperatures (53°–57° C), to tolerate much higher salt concentrations (12%–15% NaCl), to assimilate citrate for growth and to reduce nitrate to nitrite (Boyer et al. 1973; Claus and Berkeley 1986). Strains KP 1239, KP 1241, KP 1242 and KP 1243 belonging to the genus *Bacillus* all bear these four differential characteristics along with those shared commonly with *B. alcalophilus* subsp. *halodurans* and *B. alcalophilus*. This situation together with the GC content of DNA justifies the assignment of four soil isolates to strains of *B. alcalophilus* subsp. *halodurans* [GC of type strain NRRL B-3881 (ATCC 27557) = 42.6 mol%] rather than *B. alcalophilus* [GC of type strain NCTC 4553 (ATCC 27647) = 36.7 mol%] (Boyer et al. 1973; Claus and Berkeley 1986).

B. alcalophilus subsp. *halodurans* as well as *B. alcalophilus* has proteolytic activity (Boyer et al. 1973; Claus and Berkeley 1986). *B. alcalophilus* ATCC 21522 [designated as *Bacillus* sp. 221 by Horikoshi (1971)] produces a large amount of alkaline serine protease. This extracellular enzyme has already been crystallized and characterized for its molecular and catalytic properties (Horikoshi 1971). In contrast, there has never been any information on *B. alcalophilus* subsp. *halodurans* about either the feature of its protease production or the nature of its produced protease.

Crystalline alkaline protease produced by *B. alcalophilus* subsp. *halodurans* KP 1239 strikingly resembles *B. alcalophilus* ATCC 21522 protease in mol. wt., $s_{20,w}$, pI and amino acid composition, although the former pI is slightly lower than the latter (=9.2) (Table 2) (Horikoshi 1971). Their N-terminal sequences of 20 residues are absolutely identical, but only 45% and 65% homologous to the subtilisins Carlsberg and BPN′, respectively (Fig. 4). The strain KP 1239 enzyme shares its antigenic determinants with strain ATCC 21522 enzyme, but with neither those of subtilisins Carlsberg nor BPN′ (Fig. 5). These findings seem suggestive of a great deal of similarity between KP 1239 and ATCC 21522 enzymes in the shape, size, surface electric charge, and amino acid sequence. This is not surprising in the light of these enzymes being produced by the strains of closely related species, as demonstrated with neutral proteases of *B. stearothermophilus* MK 232 and *B. thermoproteolyticus* Rokko (Kubo and Imanaka 1988).

B. alcalophilus subsp. *halodurans* KP 1239 protease is a serine protease, as its activity is strongly inhibited by diisopropylfluorophosphate and PMSF. The pH and temperature for optimal activity and the stability of activity against heat are comparable to those of *B. alcalophilus* ATCC 21522 protease (Fig. 6) (Horikoshi 1971). The elevation of optimum temperature and heat stability in the presence of Ca^{2+} is a general aspect of alkaline proteases, although the optimum temperature is slightly higher for the KP 1239 enzyme than for the ATCC 21522 enzyme (60°–65° C) (Fig. 6) (Horikoshi 1971; Kelly and Fogarty 1976; Strongin et al. 1979; Tsuchida et al. 1986; Takami et al. 1989; Manachini et al. 1988).

B. alcalophilus subsp. *halodurans* KP 1239 can produce alkaline protease very efficiently from citric acid at neutral to alkaline initial pHs on simple media containing 1% citric acid and 0.3% yeast extract (Table 1, Fig. 2). The omission of citric acid results in a drastic fall in enzyme production (Table 1). Alkalophilic *Bacillus* sp. 8-1, closely related to *B. megaterium,* can produce alkaline protease from methyl acetate or acetic acid as a carbon source at alkaline pHs (Kitada and Horikoshi 1976). However, acetic acid is far less effective for alkaline protease production by *B. alcalophilus* subsp. *halodurans* KP 1239 than citric acid (Table 1). This strain releases alkaline protease after the exponential phase of growth, as observed with most extracellular *Bacillus* proteases (Debabov 1982; Ikeda et al. 1974; Kitada and Horikoshi 1976; Manachini et al. 1988) (Fig. 2). The enzyme accumulation by strain KP 1239 amounts to about 24% of extracellular proteins at 28 h cultivation, which is nearly comparable to those of alkaline proteases by alkalophilic *Bacillus* sp. strains NKS-21 and AH-101 (22% and 33%, assessed from each final enzyme purification rate over culture) (Tsuchida et al. 1986; Takami et al. 1989). Such a high content as well as the simple medium used may make it easier to purify the KP 1239 enzyme to crystalline form by using only three steps (anion and cation exchanger treatments, and crystallization).

Acknowledgement. This work was supported by grants from the Komori Memorial Foundation, Osaka, Japan.

References

Aunstrup K, Outtrup H, Andersen O, Pambmann C (1972) Proteases from alkalophilic *Bacillus* species. In: Terui G (ed) Proceedings of the 4th international symposium, Fermentation technology today Society Fermentation Technology, Osaka, Japan, pp 299–305

Boyer EW, Ingle MB, Mercer GD (1973) *Bacillus alcalophilus* subsp. *halodurans* subsp. nov.: an alkaline-amylase-producing, alkalophilic organism. Int J Syst Bacteriol 23:238–242

Claus D, Berkeley RCW (1986) Genus *Bacillus* Cohn 1872, 174^{AL}*. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 2. Williams and Wilkins, Baltimore, pp 1105–1139

Debabov VG (1982) The industrial use of bacilli. In: Dubnau DA (ed) The molecular biology of the bacilli. Academic Press, New York, pp 331–370

Delange RJ, Smith EL (1968) Amino acid composition; isolation and composition of peptides from the tryptic hydrolysate. J Biol Chem 243:2134–2142

Fujiwara N, Yamamoto K (1987) Production of alkaline protease in a low-cost medium by alkalophilic *Bacillus* sp. and properties of the enzyme. J Ferment Technol 65:345–348

Horikoshi K (1971) Production of alkaline enzymes by alkalophilic microorganisms; part I. Alkaline protease produced by *Bacillus* No 221. Agric Biol Chem 35:1407–1414

Horikoshi K, Akiba T (1982) Alkalophilic microorganisms, a new microbial world. Japan Scientific Societies Press, Toyko; Springer, Berlin, Heidelberg, New York, pp 93–101

Ikeda S, Tobe S, Niwa K, Ishizaki A, Hirose Y (1974) Production of alkaline protease from acetic acid. Agric Biol Chem 38:2317–2322

Kelly CT, Fogarty WM (1976) Microbial alkaline enzymes. Process Biochem 11:3–9

Kitada M, Horikoshi K (1976) Alkaline protease production from methyl acetate by alkalophilic *Bacillus* sp. J Ferment Technol 54:383–392

Kubo M, Imanaka T (1988) Cloning and nucleotide sequence of the highly thermostable neutral protease gene from *Bacillus stearothermophilus*. J Gen Microbiol 134:1883–1892

Lineweaver H, Burk D (1934) The determination of enzyme dissociation constants. J Am Chem Soc 56:658–666

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Manachini PL, Fortina MG, Parini C (1988) Thermostable alkaline protease produced by *Bacillus thermoruber* – a new species of *Bacillus*. Appl Microbiol Biotechnol 28:409–413

Marmur J, Doty P (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol 5:109–118

Martin RG, Ames B (1961) A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J Biol Chem 236:1372–1379

Miura K (1967) Preparation of bacterial DNA by the phenol-pH 9-RNases method. Methods Enzymol 12:543–545

Smith EL, Delange RJ, Evans WH, Landon M, Markland FS (1968) Subtilisin Carlsberg V: the complete sequence; comparison with subtilisin BPN′; evolutionary relationships. J Biol Chem 243:2184–2191

Strongin AYA, Abramov ZT, Yaroslavtseva NG, Baratova LA, Shaginyan KA, Belyanova LP, Stepanov VM (1979) Direct comparison of the subtilisin-like intracellular protease of *Bacillus licheniformis* with the homologous enzymes of *Bacillus subtilis*. J Bacteriol 137:1017–1019

Suzuki Y, Tomura Y (1986) Purification and characterization of *Bacillus coagulans* oligo-1,6-glucosidase. Eur J Biochem 158:77–83

Suzuki Y, Yuki T, Kishigami Y, Abe S (1976) Purification and properties of extracellular α-glucosidase of a thermophile, *Bacillus thermoglucosidius* KP 1006. Biochim Biophys Acta 445:386–397

Suzuki Y, Nakamura N, Mizoguchi M, Abe S (1980) Molecular properties of *Bacillus thermoglucosidius* exo-oligo-1,6-glucosidase. Sci Rep Kyoto Prefect Univ Agric 32:190–200

Takami H, Akiba T, Horikoshi K (1989) Production of extremely thermostable alkaline protease from *Bacillus* sp. no. AH-101. Appl Microbiol Biotechnol 30:120–124

Takii Y, Taguchi H, Shimoto H, Suzuki Y (1987) *Bacillus stearothermophilus* KP 1236 neutral protease with strong thermostability comparable to thermolysin. Appl Microbiol Biotechnol 27:186–191

Tsuchida O, Yamagata Y, Ishizuka T, Arai T, Yamada J, Takeuchi M, Ichisima E (1986) An alkaline protease of an alkalophilic *Bacillus* sp. Curr Microbiol 14:7–12

Ward OP (1983) Proteases. In: Fogarty WM (ed) Microbial enzymes and biotechnology. Applied Science Publishers, London, pp 251–317

Weber K, Osborn M (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244:4406–4412

Wrigley CW (1968) Analytical fractionation of plant and animal proteins by gel electrofocusing. J Chromatogr 36:362–365