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Purification of alkaline proteases from a *Bacillus* strain and their possible interrelationship

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Abstract Alkalophilic *Bacillus* sp. KSM-K16 produced three alkaline proteases, as detected by polyacrylamide gel electrophoresis (PAGE). The major protease, designated M protease, was recently purified to homogeneity and its properties were characterized. In the present study, two minor proteases, designated H protease and N protease, were purified to homogeneity from cultures of this organism. H protease had a molecular mass of 28 kDa, as estimated by sodium dodecyl sulfate/PAGE (SDS-PAGE) and its maximum activity against casein was observed at pH 11.0 and at 55°C. N protease consisted of two polypeptide chains with molecular masses of 12.5 kDa and 14.5 kDa, as estimated by SDS-PAGE, although it migrated as a single protein band during non-denaturing PAGE. Its maximum activity was observed at pH 11.0 and at 60°C. The amino-terminal sequences of H protease and of the 14.5-kDa polypeptide of N protease were identical to that of M protease. The electrophoretic relationship between the three enzymes was examined after they had been stored at different pH values and at 5°C. M protease was converted to H protease more rapidly at pH 11 than at pH 8 or below, and H protease was converted to M protease at pH 8 or below but not at pH 11. N protease appeared to be the autolytic product of the M and H proteases.

Introduction

We previously isolated a strain of alkaline-protease-producing *Bacillus* sp., designated KSM-K16, from a soil sample and succeeded in the industrial production of the enzyme using a hyperproducing mutant derivative of the original strain. Since 1991, this alkaline protease has been added to compact, heavy-duty laundry detergents. The alkaline protease, designated M protease, has been purified to homogeneity and its properties have been characterized (Kobayashi et al. 1995). We have also cloned and sequenced the gene for M protease (Hakamada et al. 1994) and have determined the three-dimensional structure of the enzyme by X-ray diffraction (Yamane et al. 1995).

During the course of purification of M protease, we found that the crude preparation of the enzyme from cultures of *Bacillus* sp. KSM-K16 contained two other alkaline proteases, as visualized by non-denaturing polyacrylamide gel electrophoresis (PAGE). In order to analyze the multiple electrophoretic forms of alkaline protease from *Bacillus* sp. KSM-K16, we purified the two minor proteases, designated H protease and N protease, to homogeneity. In this report, we describe their enzymatic properties and a possible mechanism for the interconversion of the main protease, namely M protease, and the two minor H and N proteases.

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Materials and methods

Bacterial strain and growth conditions

Bacillus sp. KSM-K16 was used as the producer of alkaline protease. The organism was grown in an alkaline medium at 30°C for 48 h (Kobayashi et al. 1995).

Purification of alkaline proteases

The culture supernatant (1 l) was obtained by centrifugation (8000 *g* for 10 min at 4°C) and concentrated to a small volume by ultrafiltration on a YM-5 membrane (Amicon). The concentrate was dialyzed overnight against 5 l 2 mM $CaCl_2$, and the retentate was then lyophilized. The residue (1.5 g) was dissolved in 20 ml 2 mM $CaCl_2$ and dialyzed overnight against 5 l 5 mM TRIS/HCl buffer (pH 8.0) that contained 2 mM $CaCl_2$. The retentate was loaded on a column

of DEAE-Bio-Gel A (2.5 cm × 16 cm; Bio-Rad), which had been equilibrated with the basal buffer, 10 mM TRIS/HCl buffer (pH 8.0) plus 2 mM $CaCl_2$. The column was washed with 150 ml basal buffer and the unabsorbed eluate containing alkaline protease activity was concentrated to a small volume (20 ml) by ultrafiltration. The concentrate was applied to a column of CM-Bio-Gel A (2.5 cm × 16 cm; Bio-Rad) that had been equilibrated with the basal buffer. The column was washed with 250 ml of the same buffer, and proteins were eluted first with a 450-ml linear gradient of KCl up to 30 mM in the basal buffer at a flow rate of 44 ml h^{-1} cm^{-2}. Fractions of 6.5 ml were collected. N protease was eluted between 10 mM and 20 mM KCl (fraction numbers 23–41) and H protease was eluted between 20 mM and 30 mM KCl (fractions 45–69). After the enzymatic activity and homogeneity had been checked in each fraction, the fractions containing the H protease and the N protease were separately combined. M protease was then eluted with a 450-ml linear gradient of 30–100 mM KCl in the basal buffer. Fractions that contained M protease activity were eluted between 35 mM and 75 mM KCl (fractions 77–101). For further purification of H protease, the appropriate pooled fractions were concentrated to 7.2 ml by ultrafiltration. The concentrate was then loaded on a column of CM-Bio-Gel A (1.6 cm × 16 cm) equilibrated with the basal buffer. The column was washed with 150 ml of the same buffer, and proteins were eluted with a 300-ml linear gradient of KCl up to 25 mM in the buffer. Fractions of 3.3 ml were collected at a flow rate of 36 ml h^{-1} cm^{-2}. Fractions containing electrophoretically pure H protease were pooled (fractions 41–44) and then washed and concentrated in the basal buffer to 1.0 ml by ultrafiltration.

The pooled fractions of N protease were concentrated to 4.3 ml by ultrafiltration and the concentrate was loaded on a column of hydroxyapatite (1.5 cm × 10 cm; Bio-Rad) that had been equilibrated with 50 mM phosphate buffer (pH 7.0) plus 0.2 mM $CaCl_2$. After the column had been washed with 50 ml equilibrating buffer, proteins were eluted with a 100-ml linear gradient of 50–200 mM phosphate buffer (pH 7.0) plus 0.2 mM $CaCl_2$. The eluate was collected in 3.0-ml fractions at a flow rate of 14 ml h^{-1} cm^{-2}, and the purity of N protease in each fraction was checked by PAGE. The active fractions, which were eluted above 180 mM phosphate buffer (fractions 28–33), were combined. The pooled N protease fractions were washed and concentrated in the basal buffer to 1.8 ml by ultrafiltration. The resultant concentrates were used exclusively for further experiments as the final preparations of purified enzyme. The purified enzymes were brought to a final concentration of 20% (v/v) glycerol and stored at −20°C when necessary.

Assays of enzymatic activity

Caseinolytic and oligopeptidyl-*p*-nitroanilide-hydrolyzing (*p*NA-hydrolyzing) activities were measured as described previously (Kobayashi et al. 1995). One unit (U) of *p*NA-hydrolyzing activity was defined as the amount of enzyme that liberated 1 μmol *p*-nitroaniline/min at 30°C and at pH 9.0 from a 5 mM solution of substrate in 50 mM TRIS/HCl buffer plus 2 mM $CaCl_2$. The measurement of caseinolytic activity was used routinely for assays of proteases. One unit of caseinolytic activity was defined as the amount of enzyme that released acid-soluble peptides equivalent to 1 μmol L-tyrosine/min at 40°C and at pH 10.0 in 50 mM borate/NaOH buffer.

Protein was quantified by the method of Lowry et al. (1951) with bovine serum albumin (Sigma) as the protein standard.

Gel electrophoresis

Non-denaturing PAGE was performed by the method of Taber and Sherman (1964), using 7.5% slab gels and 25 mM TRIS/192 mM glycine buffer (pH 8.3) as the electrode buffer. The method for staining for protease activity after PAGE was described previously (Kobayashi et al. 1995). Sodium dodecyl sulfate (SDS)/PAGE was performed with 12.5% acrylamide gels by the method of Laemmli (1970). Proteins were stained with Coomassie brilliant blue R-250 (Sigma).

Isoelectrofocusing PAGE was performed in 6% acrylamide gels supplemented with 2% (v/v) ampholyte (Pharmalyte, pH 3–10, pH 8–10.5; Pharmacia) using an isoelectric focusing cell apparatus (model 111; Bio-Rad), in accordance with the supplier's directions.

Measurements of circular dichroism

Circular dichroism spectra of the enzymes were recorded with an automatic recording spectrophotometer (model J-720; Jasco). Each enzyme, at 0.1 mg/ml in aqueous solution, was placed in a 0.4-ml cylindrical cuvette with a path length of 0.5 cm, and spectra were recorded from 260 nm to 180 nm at room temperature under a stream of nitrogen gas at 10 l/min. The content of α-helices was estimated from the mean residue ellipticity at 222 nm by the method of Yang et al. (1986) using a computer program (SSE-338; Jasco).

Amino-terminal sequences of the enzymes

H protease (30 μg) was concentrated and desalted in a centrifugal concentrator (Ultra free C3LGC; Millipore). In the case of N protease, the enzyme (39 μg) was purified by SDS-PAGE, and then transferred to a membrane (Immobilon-P; Millipore), by the method of Towbin et al. (1979). After the band of protein on the membrane had been verified by staining with Coomassie brilliant blue R-250, the gel that corresponded to the protein band was cut into small pieces. Samples were then subjected to Edman degradation with an automated sequencer (model 477A; Applied Biosystems) for determination of N-terminal amino acid sequences. Phenylthiohydantoin (PTH) derivatives of amino acids were fractionated and detected with an amino acid PTH analyzer (model 120A; Applied Biosystems).

Amino acid analysis

The amino acid composition of N protease was analyzed by the same procedure as described previously (Kobayashi et al. 1995) using an amino acid autoanalyzer (model L-8500; Hitachi). Measurements of tryptophan and cysteine were performed by the methods of Edelhoch (1967) and Yoshida et al. (1977) respectively.

Cleavage of proteases by cyanogen bromide (CNBr)

M protease was purified to homogeneity as described previously (Kobayashi et al. 1995). The conversion of M to H protease or H to M protease, distinguishable by PAGE, was done by storage at 5°C for 2 weeks either in 50 mM carbonate buffer (pH 11) or in 50 mM acetate buffer (pH 6) plus 2 mM $CaCl_2$ respectively. The gels for protease bands obtained after PAGE were cut into small pieces. They were placed in small glass bottles, to which was added 1 ml each of 1.5% (w/v) CNBr (Wako Pure Chemical) in 70% (v/v) formic acid. After the headspace of the bottle had been filled with nitrogen gas, they were stored at room temperature for 41 h with gentle stirring. The gel debris and excess solvent were removed by filtration and then centrifugal evaporation (model CVE-200D; Tokyo Rikakikai) respectively. The dried materials were washed twice with distilled water, dried by centrifugal evaporation, and then redissolved in 100 μl distilled water. The CNBr-cleaved peptides thus obtained were fractionated by HPLC on a column of DEAE-5PW

Table 1 Summary of the purification of H and N proteases

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude enzyme	79 560	918	86.7	100
DEAE-Bio-Gel A	45 000	620	72.6	56.6
CM-Bio-Gel A				
H protease	7 020	108	65.0	8.8
N protease	3 240	68.9	47.0	4.1
M protease[a]	16 450	257	64.0	20.7
CM-Bio-Gel A				
H protease	408	3.4	120.0	0.51
M protease[a]	14 770	116	127.3	18.6
Hydroxyapatite				
N protease	33.2	8.8	3.8	0.04

[a] Kobayashi et al. (1995)

(7.5 × 75 mm; Tosoh) equipped with a pump (model LC10AD) and a UV detector (model SPD10A; Shimazu). They were eluted with a 40-min linear gradient at 1.0 ml/min from 10 mM TRIS/HCl buffer (pH 8.0) to the same buffer plus 0.8 M NaCl at room temperature. The eluted peptides were monitored spectrophotometrically at 220 nm. The CNBr-cleaved peptides were also separated by SDS-PAGE on 24% (w/v) acrylamide gels.

Preparation of recombinant M protease

Bacillus subtilis ISW1214 (pHA114), harboring the gene for M protease (Hakamada et al. 1994), was propagated at 30 °C for 3 days, with shaking, in PY medium supplemented with 15 μg/ml tetracycline. PY medium contained (w/v) 2.0% polypeptone (Nihon Pharmaceutical), 0.1% yeast extract (Difco), 0.1% KH_2PO_4, and 0.5% NaCl (pH 6.8). After removal of cells by centrifugation (8000 *g*, 15 min) at 4 °C, the supernatant was concentrated and washed with 10 mM TRIS/HCl buffer (pH 8.0) plus 2 mM $CaCl_2$ by ultrafiltration on a YM-5 membrane. To the concentrate (7 ml) was added 2 ml DEAE-Bio-Gel A that had been equilibrated with the same buffer. After gentle stirring for 5 min, the resin was removed by filtration and the filtrate was concentrated again by ultrafiltration. The concentrate was subjected to PAGE and protease activities in the gel were visualized by activity staining (Kobayashi et al. 1995).

Results

Purification and homogeneity of the alkaline proteases

A summary of a typical purification of the proteases is shown in Table 1. The specific activities against casein of H and N proteases were 120.0 U/mg and 3.8 U/mg respectively. Very low specific activity was obtained for N protease, but the specific activity of H protease was almost identical to that of M protease (127 U/mg) (Kobayashi et al. 1995). The preparations of purified proteases were homogeneous, as judged from their migration as single bands of protein during PAGE (Fig. 1A).

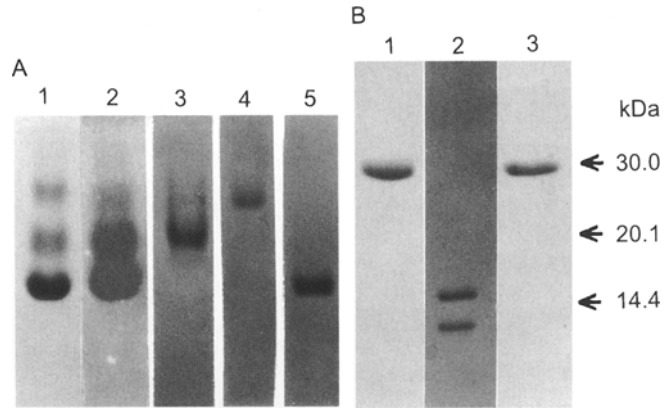

Fig. 1A, B Polyacrylamide gel electrophoresis (PAGE). **A** Non-denaturingPAGE. Lanes: *1* crude enzyme (35 μg), *2* activity staining of crude enzyme, *3* purified H protease (10 μg), *4* purified N protease (10 μg), *5* purified M protease (10 μg). **B** SDS-PAGE. Enzymes had been preincubated with 1 mM phenylmethylsulfonyl fluoride (PMSF) at 30 °C for 20 min to avoid autolysis, and then treated at 100 °C for 5 min with 2.3% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol according to the method of Laemmli (1970). *1* Purified H protease (10 μg), *2* purified N protease (10 μg), *3* purified M protease (10 μg). Molecular mass markers: carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa)

Molecular masses and isoelectric points

The molecular mass of H protease was estimated to be 28 kDa by SDS-PAGE. N protease was composed of two polypeptides with molecular masses of 12.5 kDa and 14.5 kDa (Fig. 1B). The isoelectric point was estimated to be about pH 10.6 for H protease and around pH 10.0 for N protease from isoelectrofocusing PAGE (data not shown).

Amino-terminal sequences and α-helix contents

The amino-terminal sequences of H protease and that of the 14.5-kDa fragment of N protease were both Ala-Gln-Ser-Val-Pro-Trp-Gly-Ile, which were identical with that of M protease (Kobayashi et al. 1995). Determination of the N-terminal amino acid sequence of the 12.5-kDa polypeptide of N protease was unsuccessful because of autodigestion during transfer to a blotting membrane from SDS-PAGE.

The α-helix contents of H and N proteases were calculated to be 26.3% and 18.9% respectively, from the CD spectrometric data.

Effects of pH and temperature on enzymatic activities

The effects of pH on enzymatic activities against casein at 40 °C in various buffers, from pH 4.1 to pH 13.2, are

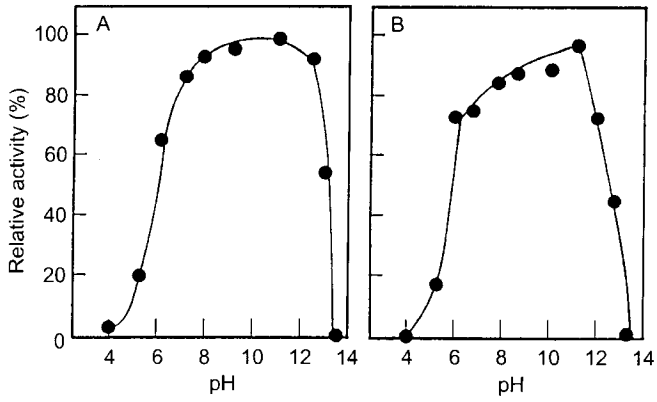

Fig. 2A, B Effect of pH on enzymatic activities. The enzymatic activity was measured in a total reaction volume of 1.1 ml that contained 0.34 μg H protease (**A**) or 13.8 μg of N protease (**B**) in the following buffers (50 mM) : acetate (pH 4.0–6.2), phosphate (pH 6.3–8.5), carbonate (pH 8.9–11.0), phosphate/NaOH (pH 11.0–12.3) and KCl/NaOH (pH 12.0–13.2)

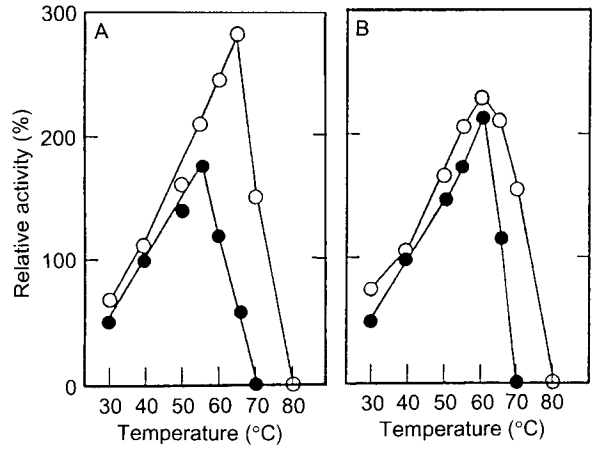

Fig. 3A, B Effect of temperature on enzymatic activities. Purified H protease (0.23 μg) or N protease (9.8 μg) was added to a 1% (w/v) solution of casein in 50 mM borate/NaOH buffer (pH 10.0). After a 10-min incubation at the given temperature, the reaction was stopped by addition of trichloroacetic acid. The enzymatic activities are expressed percentages of the activity at 40 °C in the absence of $CaCl_2$. **A** H protease, **B** N protease. ○The relative activity in the presence of 5 mM $CaCl_2$, ●that in the absence of $CaCl_2$

shown in Fig. 2. The optimum pH values for the reactions of H and N proteases were pH 11.0 each in carbonate buffer. The effects of temperature on enzymatic activity against casein in 50 mM borate/NaOH buffer (pH 10.0) are shown in Fig. 3. The optimum temperatures for the reactions catalyzed by H and N proteases were about 55 °C and 60 °C respectively. In the presence of 5 mM $CaCl_2$, the temperature for maximum activity of H protease shifted to 65 °C and the activity was 1.5-fold greater than that observed at 55 °C in the absence of $CaCl_2$. By contrast, the effect of $CaCl_2$ was not very conspicuous in the case of N protease.

Effects of pH and temperature on stability of the enzymes

The enzymes were very stable to incubation at 55 °C for 10 min in buffers from pH 5.0–12.0, when buffers were supplemented with 2 mM $CaCl_2$. In the absence of $CaCl_2$, both enzymes were less stable between pH 6.0 and pH 12.0, as shown in Fig. 4. To examine the thermal stability of H and N proteases, the enzymes were incubated at various temperatures for 10 min in 20 mM borate/NaOH buffer (pH 9.0) in the absence and presence of 5 mM $CaCl_2$. In the absence of $CaCl_2$, H and N proteases were both stable up to 50 °C. When $CaCl_2$ was present, H and N proteases were stable up to 60 °C and 55 °C respectively (Fig. 5).

Effects of various cations and chemical reagents

H and N proteases were incubated with various metal ions (1 mM) at 30 °C for 20 min in 20 mM borate/

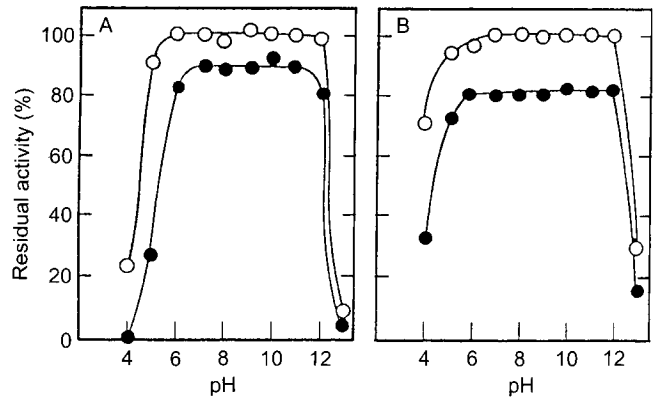

Fig. 4A, B Effect of pH on the stability of H protease (**A**) and N protease (**B**). Purified H protease (14.2 μg) or N protease (447 μg) was added to buffers of various pH values. After a 10-min preincubation at 55 °C, the solutions of treated enzymes were put in an ice bath and diluted with 50 mM borate/NaOH (pH 10.0), and then the residual activity was measured. The following buffers (20 mM) were used: acetate (pH 4.0–6.0), phosphate (pH 6.0–8.0), carbonate (pH 9.0–11.0), phosphate/NaOH (pH 11.0–12.5) and KCl/NaOH (pH 11.0–12.5). The original enzymatic activity before the preincubation at various pH values was taken as 100%. ○The residual activity in the presence of 2 mM $CaCl_2$, ●that in the absence of $CaCl_2$

NaOH buffer (pH 9.0). The residual activities were then measured under the standard assay conditions. The following cations had almost no effect on the activities of both enzymes: Co^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, and Al^{3+} ions. H protease was inhibited by Hg^{2+} ion (48%) and N protease by Hg^{2+} (20%) and Cu^{2+} ions (13%).

The effects of various inhibitors on the activities of the two enzymes were examined at 30 °C and at pH 7.0

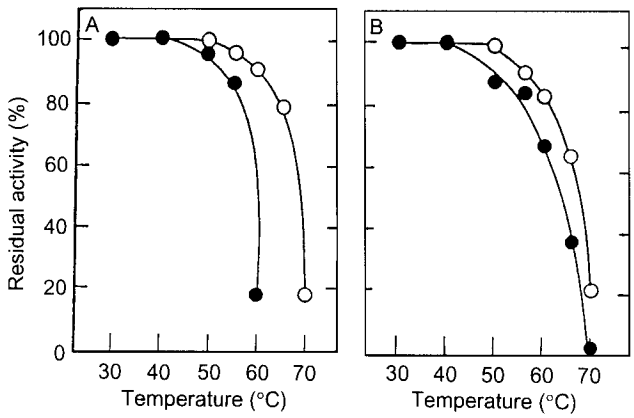

Fig. 5A, B Effect of temperature on the stability of H protease (**A**) and N protease (**B**). Purified H protease (3.4 μg) or N protease (632 μg) had been preincubated at various temperatures in 20 mM borate/NaOH buffer (pH 9.0). After a 10-min preincubation at the indicated temperature, the solutions of treated enzymes were put in an ice bath and diluted with 50 mM borate/NaOH buffer (pH 10.0). The residual activity was then measured. The enzymatic activity, after preincubation at 30 °C for 10 min, was taken at 100%. ○The residual activity in the presence of 5 mM $CaCl_2$,●that in the absence of $CaCl_2$

in 20 mM phosphate buffer. Phenylmethylsulfonyl fluoride (1 mM) and chymostatin (60 ppm) inhibited the H protease activity by 97.5% and 58% respectively, and they inhibited the N protease activity by 97.9% and 62% respectively. H protease was inhibited to a moderate extent by incubation at 30 °C for 2 h with the following reagents (2 mM); *N*-tosyl-L-phenylalanine chloromethylketone (30%) and *N*-benzyloxycarbonyl- L-phenylalanine chloromethyl ketone (38%). N protease was less sensitive to these reagents (26% and 16% inhibition respectively). Neither enzyme was inhibited at all by *p*-chloromercuribenzoate (1 mM), dithiothreitol (5 mM), *N*-ethylmaleimide (5 mM), EDTA (5 mM), 2-hydroxy-5-nitrobenzyl bromide (5 mM), 1,2-epoxy-3-(*p*-nitrophenyl)-propane (5 mM), antipain (0.1 mM), leupeptin (0.1 mM), bestatin (0.1 mM), phosphoramidon (0.1 mM) or elastatinal (0.1 mM). These results suggest that both enzymes belong to the class of alkaline serine proteases.

The effects of various surfactants on the stability of the two enzymes were examined after the enzymes had been incubated with them (5%, w/v, each) in 0.1 M TRIS/HCl buffer (pH 9.0) at 40 °C for 4 h. The residual activities of H protease were 99% for sodium polyoxyethylene alkyl sulfonate (SAS), 67% for sodium α-olefin sulfonate (AOS), 78% for sodium alkane sulfonate (AS), 95% for α-sulfo-fatty acid ester (SFE), and 101% for softanol 70H (S70), whereas they were 37% for sodium linear alkylbenzene sulfonate (LAS), and 38% for SDS. The residual activities of N protease were 72% for LAS, 100% for SAS, 86% for SDS, 95% for AOS, 95% for AS, 108% for SFE, and 108% for S70.

Substrate specificities

Among proteins tested, casein was the best substrate for both enzymes. They also hydrolyzed scleroproteins effectively. The rates of hydrolysis of keratin from animal hair (Kanto Chemicals), α-keratin from human skin (Hattori and Ogawa 1980) and elastin from bovine neck ligament (Sigma) were 57%, 65%, and 14% for H protease, and 43%, 78%, and 13% for N protease respectively, when the rate of hydrolysis of casein by either enzyme was taken as 100%.

With the various oligopeptidyl-*p*NA substrates tested, H and M proteases were most active against *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, as summarized in Table 2. It is of interest that the k_{cat}/K_m values for *N*-succinyl-Ala-Ala-Pro-Met-*p*NA and methoxy succinyl-Ala-Ile-Pro-Met-*p*NA of H protease were 10- to 20-fold lower than those of M protease. However, the differences in apparent K_m values of both the substrates for H and M proteases were not large. The catalytic efficiency and other kinetic parameters of N protease for reactions with these synthetic substrates were not examined because this enzyme exhibited very low specific activity compared with those of H and M proteases.

Table 2 Kinetic parameters of the *p*-nitroaniline-liberating activity of H and M proteases from *p*NA substrates. *Suc N*-succinyl, *Suc(OMe) N*-methoxysuccinyl, *Cbz N*-benzyloxycarbonyl, *Cte* citrulline

Substrate	H protease			M protease[a]		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Suc-AAPF	0.60	74.7	125.5	0.80	139.5	174.4
Suc-AAPM	0.71	5.5	7.7	1.01	138.7	137.3
Suc-AAVA	0.15	6.5	44.8	0.09	11.9	129.3
Suc(OMe)-AIPM	0.89	4.0	4.5	2.19	98.4	44.9
Cbz-GPCi	1.02	22.6	22.2	0.74	26.4	35.9
Suc-AAPL	2.04	32.6	16.0	3.23	67.2	20.8
Suc-AAA	2.40	14.4	6.0	2.22	17.9	8.1

[a] Kobayashi et al. (1995)

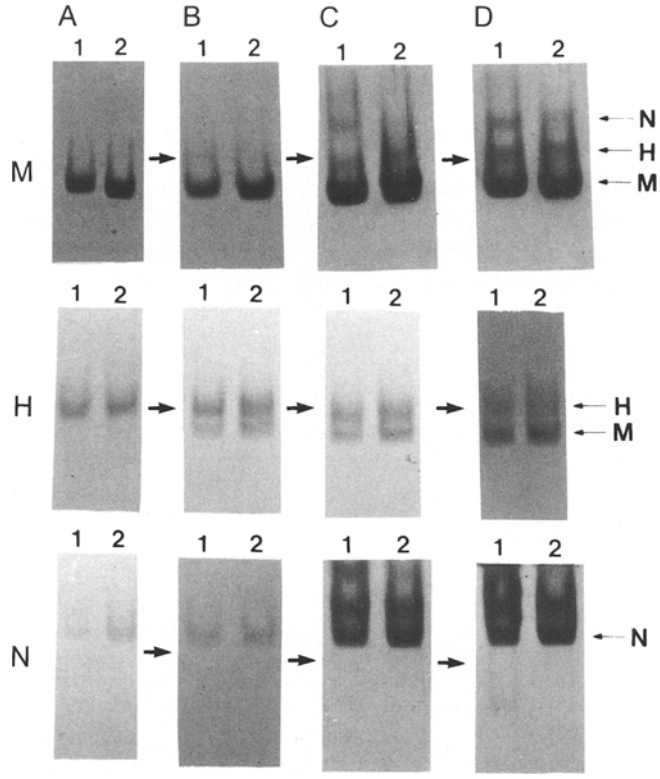

Fig. 6A–D The relationship among the multiple forms of alkaline protease. Purified M protease (*M*), H protease (*H*) and N protease (*N*) were stored in 50 mM TRIS/HCl buffer (pH 8.0) that contained 2 mM $CaCl_2$ at 5 °C for 2 months. **A** Starting materials, **B** stored for 1 week, **C** stored for 1 month, **D** stored for 2 months. *1* Stored in the absence of PMSF, *2* stored in the presence of 2 mM PMSF. Protein bands (10 μg protein/lane for M and H proteases, 4.5 μg for N protease) were stained with Coomassie brilliant blue R-250. **C**, **D** Excess N protease (14 μg) was used and stained by a silver stain kit (Kanto Chemicals)

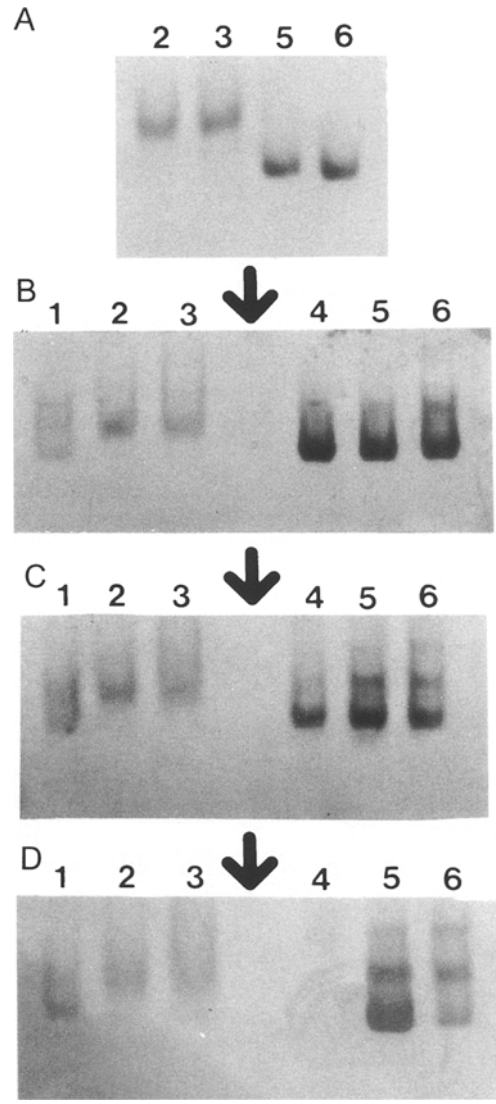

Fig. 7A–D Effect of pH on the interconversion of the multiple forms of alkaline protease. Purified M protease and H protease were stored in several buffers (50 mM) plus 2 mM $CaCl_2$ and 2 mM PMSF at 5 °C for a month. **A** Starting material, **B** stored for a week, **C** stored for 2 weeks, **D** stored for a month. Lanes: *1* and *4* acetate buffer (pH 5.0), *2* and *5* carbonate buffer (pH 11.0), *3* and *6* phosphate/NaOH buffer (pH 11.0). *1–3* H protease, *4–6* M protease

Relationship between the three proteases

The relationship between the purified M, H, and N proteases was examined by PAGE after each had been stored at 5 °C for 2 months in 50 mM TRIS/HCl buffer (pH 8.0) that contained 2 mM $CaCl_2$ in the absence and in the presence of 2 mM phenylmethylsulfonyl fluoride (PMSF). As shown in Fig. 6, M protease was gradually converted to H and N proteases when it was stored for more than a month even in the presence of PMSF. H protease was converted to M protease much more rapidly than to N protease during the course of storage for a week in the presence of PMSF. N protease was converted neither to M protease nor to H protease and it generated two new polypeptides that migrated slowly during PAGE (Fig. 6). Considering the α-helix contents of M protease (21.3%; Kobayashi et al. 1995) and H protease (26.3%) and the rapid conversion of H protease to M protease at pH 8.0, we postulated that H protease could not be converted to M protease at higher pH values, namely pH values higher than pK_a values of certain amino acid residues. Indeed, as shown in Fig. 7, H protease was not converted to M protease at alkaline pH (pH 11.0). During storage of H protease at pH 5.0, the enzyme was converted to M and N proteases. By contrast, M protease was converted to H and N proteases more rapidly at pH 11.0.

Purified M and H protease and the corresponding two protein bands, generated by incubation of M protease at pH 6 or H protease at pH 11, were each fragmented by treatment with CNBr (see Materials and methods). HPLC on a DEAE-5PW column revealed that all the CNBr-treated samples were separated into four identical, main peptides, in addition to peptide

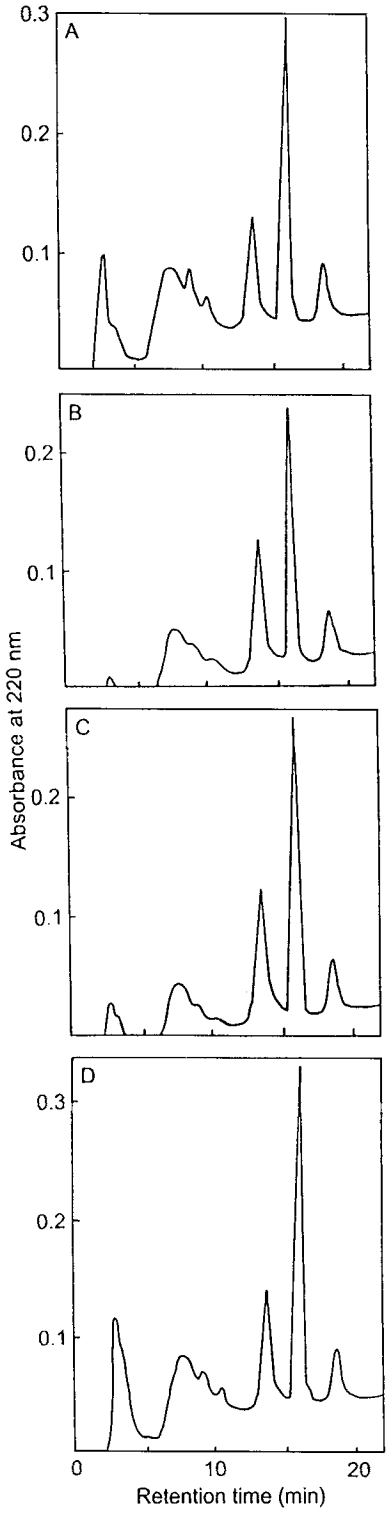

Fig. 8A–D The HPLC patterns of cleavage of proteases by CNBr. Each protein was cleaved by CNBr and subjected to HPLC on a column of DEAE-5PW with 10 mM TRIS/HCl (pH 8.0) as elution buffer, as described in Materials and methods. The elution profile of CNBr-cleaved enzyme: **A** M protease, **B** H protease, **C** the protein generated from M protease at pH 6, **D** the protein generated from H protease at pH 11

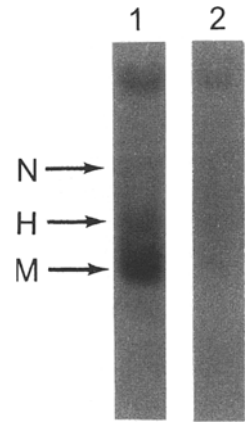

Fig. 9 Active staining of recombinant M protease expressed in *B. subtilis* ISW1214 (pHA114) cells. The methods for propagation of *B. subtilis* and purification of recombinant M protease are described in Materials and methods. *Arrows* the positions of protein bands on PAGE (7.5% acrylamide gel) for M, H and N proteases stained with Coomassie brilliant blue R-250. *1* Active staining of recombinant M protease from *B. subtilis* harboring pHA114, *2* that of the enzyme preparation from the organism harboring pHY300PLK (as control)

peaks of the remaining native enzymes (at a retention time of 3.1 min), as shown in Fig. 8. Separation of these cleaved fragments by commonly employed HPLC on an octadecyldimethylsilyl column was unsuccessful because the fragments were all tightly bound to the column and only eluted by organic solvents, such as acetonitrile and methanol. The SDS-PAGE profiles of these cleaved samples coincided well with each other. In addition, the amino acid composition of purified N protease was almost same as that of M protease (data not shown).

When the recombinant M protease was purified partially from cultures of *B. subtilis* ISW1214, harboring pHA114 (Hakamada et al. 1994), three active proteases, corresponding to M, H and N proteases, could also be detected by active staining after non-denaturing PAGE of the recombinant enzyme, as shown in Fig. 9. Since the monocistronic mRNA for M protease is expressed by the vector pHA114, the result suggests that H and N proteases have an amino acid sequence, which is identical with that of M protease.

Discussion

In the present study, two minor alkaline proteases, designated H protease and N protease, have been purified to homogeneity, as judged by PAGE, from the culture broth of *Bacillus* sp. KSM-K16, and their enzymatic properties characterized. When the culture broth was concentrated, both H and N proteases were clearly detected by activity staining after PAGE. When the level of each protease was estimated in terms of the concentration of protein (A_{280}) from the elution profile

after chromatography on a CM-Bio-Gel A column, the relative levels of M, H and N proteases were approximately 70:20:10. Such multiple electrophoretic forms of alkaline proteases are also observed in the case of other commercially available laundry alkaline proteases known as Savinase, Esperase (Novo), and Maxacal (Gist-brocades) and in the case of the crude preparation of 221 protease from *Bacillus alcalophilus* ATCC 21522 (data not shown). Zuidweg et al. (1972) also detected three protease components in a detergent enzyme, Maxatase, during isoelectrofocusing PAGE of the enzyme.

The N-terminal amino acid sequences of H protease and of the 14.5-kDa polypeptide of N protease were identical to that of M protease. Polyclonal antibodies raised against the purified M and N proteases reacted with all three enzymes in Western blotting analysis (data not shown). The molecular mass, isoelectric point and other enzymatic properties of H protease were similar to those of M protease (Kobayashi et al. 1995), with the exception of some differences in kinetic parameters for synthetic oligopeptidyl-*p*NA substrates. N protease, having a very low specific activity, was composed of two polypeptides with molecular masses of 12.5 kDa and 14.5 kDa, although this enzyme migrated as a single band of protein during non-denaturing PAGE and the amino acid composition of the enzyme was almost same as that of M protease. An N-terminal amino acid of the 12.5-kDa polypeptide was found to be arginine from the result of N-terminal sequencing of non-denatured N protease (data not shown). From an analysis of the deduced amino acid sequence (Hakamada et al. 1994), M protease (H protease) might be cleaved on the amino side of Arg-164 by autolysis, thereby producing the two polypeptides with molecular masses of 14.5 kDa and 12.5 kDa and very low specific activity.

Electrophoretic analysis suggested that M protease was gradually converted to H protease during storage at pH 8.0 and 5 °C for 2 months. The conversion, with or without phenylmethylsulfonyl fluoride, was faster at higher pH conditions, for example, at pH 11.0. H protease was converted to M protease within a week when incubated at or below pH 8.0 and 5 °C. This conversion was not observed at pH 11.0. The multiple form, reversibly convertible by change in pH, has also been found in two acid phosphatase components of rice grains (Yamagata et al. 1979). There are several examples of electrophoretic heterogeneity of enzymes, such as the serine protease of *Aspergillus sojae* (Ichishima et al. 1986), cytochrome *c* from beef heart (Flatmark 1964, 1966) and rat (Flatmark and Sletten 1968), and human carbonic anhydrase (Funakoshi and Deutsch 1969). The multiple forms of these enzymes are the result of non-enzymatic, irreversible deamination of glutamine or asparagine residues in the protein molecules. Makino et al. (1983) reported, working with protease from a strain of marine *Pseudomonas*, that a protease with high molecular mass (50 kDa) changed to a smaller protease (14.5 kDa) by autodigestion during incubation in artificial sea water.

The α-helix contents of M and H proteases are different and this conformational difference could represent the destabilization or stabilization of an α-helical structure by some amino acid residues since the conversion from H protease to M protease was not observed at pH 11.0, which is slightly higher than the pK_a values of lysine and tyrosine residues. The thiol group of cysteine also has a high pK_a value, but no cysteine residues are found in M protease (Kobayashi et al. 1995; Hakamada et al. 1994). We assume, therefore, that lysine and/or tyrosine residue(s) may be related to the conformational difference between H and M proteases at dif ferent pH values, which results in the considerable differences in k_{cat}/K_m values for synthetic substrates, such as *N*-Suc-Ala-Ala-Pro-Met-*p*NA and Suc(OMe)-Ala-Ile-Pro-Met-*p*NA. In order to explain the detailed mechanism of such interconversion, we are now replacing some of these amino acid residues in M protease with other amino acids by site-directed mutageneis using a host/vector system of *B. subtilis*, and the results will shortly be published elsewhere.

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