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Purification and Characterization of an Alkaline Protease from *Bacillus licheniformis* NS70

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A bacterial strain NS70 producing an alkaline protease was isolated from soil samples taken near a hot spring and identified as *Bacillus licheniformis* by its morphological and physiological properties and cellular fatty acid analysis. The isolated alkaline protease was purified by ammonium sulfate fractionation, DEAE-, CM-, and Phenyl-Sepharose column chromatography. The molecular weight of the purified enzyme was estimated to be 32,000 Da by sodium dodecylsulfate polyacrylamide gel electrophoresis. Its optimal pH and temperature for proteolytic activity against Hammarsten casein were 12 and 65°C, respectively. The enzyme was stable at alkaline pH range from 6.0 to 12.0, and fairly stable up to 65°C. The enzyme was inhibited by phenylmethylsulfonyl fluoride but not by EDTA and N-ethylmaleimide indicating that the enzyme is serine protease. Enzyme activity was markedly inhibited by Hg^{2+} and Cu^{2+}. Autolytic phenomena were observed on purified protease NS70 but autolysis was reduced by the addtion of Ca^{2+} ion or bovine serum albumin.

Many microbial proteases are important to the worldwide enzyme market and are used widely in the detergent, pharmaceutical and food processing industries (7). Above all, they are used extensively as a component for hydrolysis of protein stains such as blood in laundry detergents. For detergent enzymes, high activity and stability at alkaline pH, and resistance to oxidizing and chelating agents are pre-requisites. Various alkaline proteases are produced from genus *Bacillus* (4, 6, 17), *Streptomyces* (11), *Aspergillus* (13), and *Thermoactinomyces* (10). In particular, alkaline proteases secreted by both neutrophilic and alkalophilic *Bacilli* are of interest because they have been commercially used in large market as well as studied in detail. The representative proteases are subtilisins (EC 3.4.21.14) including subtilisin BPN from *Bacillus amyloliquefaciens* (16), subtilisin Carlsberg from *B. licheniformis* (9), and subtilisin E from *B. subtilis* 1168 (18). These proteases have a high optimum pH ranging from 9 to 11, and are called serine protease since they are sensitive to phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP) which react with serine residue at the active site in the enzyme. In general, these enzymes have high isoelectric points and are stabilized by Ca^{2+} ion.

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Proteases with high activity and stability in the range of alkali and high temperature are interesting for bioengineering and biotechnological applications as well as protein chemistry. Alkaline serine proteases from alkalophiles are generally stable in the highly alkaline conditions, but usually unstable at high temperatures. On the other hand, proteases from thermophiles are generally stable with heat but unstable in high pH (10). There are only a few reports concerning thermostable alkaline protease (17), although many thermostable neutral proteases from thermophiles have been reported (5, 10, 14).

Recently, we isolated a thermophilic bacterial strain NS70 producing a thermostable alkaline protease from soil sample near a hot spring. This paper deals with the identification of bacterial strain NS70 and the purification of the extracellular protease and characterization of its biochemical properties.

MATERIALS AND METHODS

Isolation and Cultivation of Alkaline Protease-Producing Bacteria

Soil samples taken near a hot spring at Yusung were suspended in sterilized water and the supernants were spreaded on solid Y medium (1% polypeptone, 0.5% yeast extract, 0.2% glycerol, 0.3% NaCl, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.01% $MgSO_4$, and 1.5% agar, pH 7.2)

containing 0.8% skim milk. Skim milk was autoclaved separately at 100°C for 10 min. The plates were incubated at 55°C overnight and colonies having large halos were selected. After cultivation of selected strains in liquid Y medium, alkaline protease activities were tested in alkaline condition (pH 10) using Hammarsten casein as a substrate. For the production of enzyme, isolated *Bacillus* sp. NS70 was cultured for 14 h at 50°C on a rotary shaker in a medium containing 1% phytone-peptone, 0.2% soluble starch, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.01% $MgSO_4$, and 0.3% NaCl (pH 7.2).

Enzyme Assay

Proteolytic activity was assayed by the method of Takami (17). Protease activity was assayed at 55°C with Hammarsten casein (Merck, Germany) as a substrate. Three milliliters of 0.6% (w/v) Hammarsten in 10 mM glycine-KCl-KOH buffer (pH 10.0) was preincubated for 10 min at 55°C and the reaction was started by the addition of 0.5 ml of the enzyme solution. After 15 min, the reaction was stopped by adding 3.0 ml of TCA mixture (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid). The reaction mixture was then kept at 4°C for 10 min and filtered through Whatman No. 5 filter paper. The absorbance of the filtrate was measured at 275 nm. One unit of the protease activity was defined as the amount of enzyme required to increase optical density at 275 nm of 0.01 in 1 min at 55°C.

Protein Measurement

The protein concentration was measured by the Bradford method (3) using dye reagent purchased from Bio-Rad. Bovine serum albumin was used as a standard.

Purification Procedures

All procedures were performed at 4°C. Solid ammonium sulfate was added to 500 ml culture supernatant to 30% saturation and left overnight. The supernatant was collected by centrifugation and brought to 80% saturation in the same manner. The precipitate was collected by centrifugation and dissolved in 50 mM Tris-HCl, pH 8.0 and dialyzed overnight against the same buffer. The dialysate was applied to DEAE-Sepharose CL-6B column (2.2 by 17 cm). The active fractions were eluted with the same buffer, pooled and concentrated by ultrafiltration with an Amicon PM 10 membrane. The enzyme solution was applied to CM-Sepharose CL-6B column previously equilibrated with 20 mM potassium phosphate buffer (pH 6.5). After the column was washed with the same buffer the protease was eluted with a linear gradient from 0 to 0.5 M NaCl. Ammonium sulfate was added to the combined active fractions to the final concentraton of 0.5 M and enzyme solution was applied to a Phenyl-Sepharose CL-4B column (1.5 by 5 cm) equilibrated with 0.5 M ammonium sulfate in 10 mM potassium phosphate buffer, pH 7.5. After the column was washed with the same buffer and with a linear gradient from 0.5 to 0 M ammonium sulfate, the enzyme was eluted with 10 mM potassium phosphate buffer, pH 7.5. The active fractions were pooled, concentrated, and used as purified enzyme.

Polyacrylamide Gel Electrophoresis

The SDS-polyacrylamide gel electrophoresis was performed in 12% slab gel as described by Laemmli (12). Before boiling at 100°C for 5 min, 10 mM PMSF was added to the enzyme solution with sample buffer. Gels were stained with Coomassie Brilliant Blue R-250. The following proteins were used as standards: bovine serum albumin (MW=66,000), hen egg albumin (MW=45,000), carbonic anhydrase (MW=29,000), lactalbumin (MW=14,200).

Effect of pH on Proteolytic Activity

To determine pH stability of the enzyme, 15 μl of enzyme solution was added to 585 μl of 0.1 M sodium acetate (pH 4-6), potassium phosphate (pH 6-7.5), Tris-HCl (pH 7.5-9) and glycine-KCl-KOH (pH 9-13) buffers. After incubation for one hour at room temperature, the remaining protease activity was measured using 70 μl of each enzyme solution as described above. To find the optimum pH of the enzyme, protease activities were checked at various pHs.

Effect of Temperature on Proteolytic Activity

The optimum temperature for enzyme activity was determined by assaying protease activities at various temperatures. To test the effect of temperature on the stability of the enzyme, enzyme solutions were incubated at various temperatures for 10 min, centrifuged and residual activities of the supernants were assayed.

Fluorescence Spectroscopy

Fluorescence measurement was performed with a Jasco FP-770 spectrofluorometer, equipped with a thermostat-controlled cell holder. Denaturation and autolysis were monitored by following the decrease in fluorescence intensity at 306 nm emission wavelength after an excitation wave length at 275 nm with a heating rate of 1°C/min.

N-terminal Amino Acid Sequencing

Purified protein was subjected to SDS-PAGE and electroblotted on polyvinylidene difloride membrane (Bio-Rad Trans-Blot Transfer Medium). After staining with Coomassie Brilliant Blue R-250, protein bands were cut out. The N-terminal amino acid sequence was determined by the automated Edman method using a gas-phase protein sequencer (model 476A, Applied Biosystem Ins., Foster, CA, USA).

RESULTS AND DISCUSSION

Isolation and Taxonomic Characterization of Strain NS70

Several isolates with large halos on skim milk plate

Table 1. Taxonomical properties of strain NS-70.

From: Rods
Motility: Motile
Spore: Ellipsoidal spore
Gram stain: Positive
Gelatin liquefaction: Positive
Indole production: Negative
Urease activity: Negative
Nitrate reduction to nitrite: Positive
Nitrite reduction to N_2: Negative
Utilization of saccharides
glucose, mannose, arabinose, maltose: Positive
caprate, adipate, phenyl-acetate: Negative
Growth temperature: 50-55°C
pH for cell growth: 7.0-8.0

Table 2. Cellular fatty acid profile of strain NS70.

Fatty acid	Contents (%)
14 : 0 iso	0.59
14 : 0	0.50
15 : 0 iso	37.51
15 : 0 anteiso	27.64
16 : 1 w7c alcohol	0.96
16 : 0 iso	2.04
16 : 1 wllc	2.08
16 : 0	2.66
iso 17 : 1 w10c	4.48
17 : 1 iso I/anteiso B	2.12
17 : 0 iso	10.38
17 : 0 anteiso	9.04

were selected. Among them, a bacterial strain NS70 showed alkaline protease activity. The taxonomic characteristics of this strain are summarized in Table 1. The strain NS70 was aerobic, spore-forming, gram positive, motile, and rod-shaped. Identification of the strain NS70 was carried out according to Bergey's Manual of Determinative Bacteriology (8) and fatty acid analysis. On the analysis of cellular fatty acid composition, predominant fatty acids were 15 : 0 iso, and 15 : 0 anteiso (Table 2). Comparison of these results with the MIDI system (15) indicated that isolate NS70 was a strain of *Bacillus licheniformis* and named *Bacillus licheniformis* NS70.

Purification of NS70 Protease

The protease was purified from the culture supernatant of *B. licheniformis* NS70 as described in Materials and Methods. Most of the colored material in the crude enzyme solution as well as 75% of the proteins were absorbed onto DEAE-Sepharose column, and protease activities were detected at the washed-out fractions (Fig. 1A). The active fractions were pooled, concentrated by ultrafiltration, and applied to CM-Sepharose column. After a linear gradient was applied to the column, protease was eluted at 0.2 M NaCl (data not shown). Finally, the

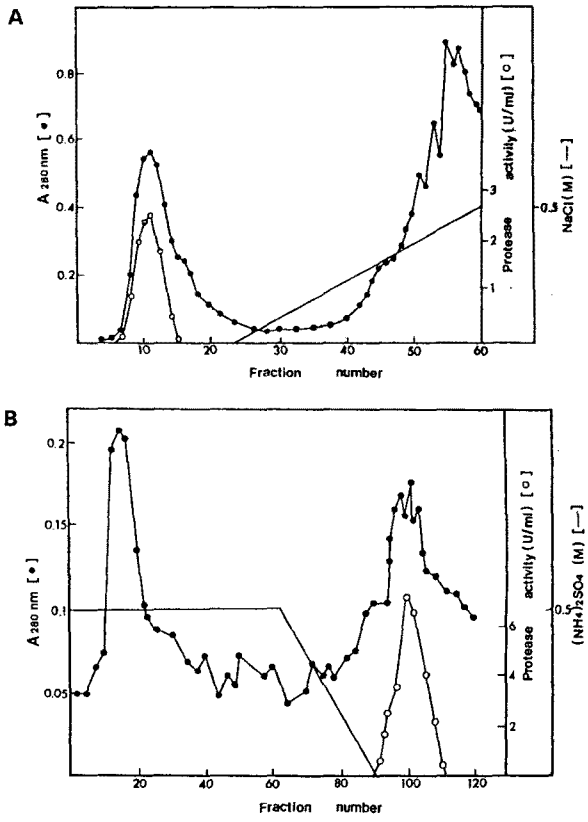

Fig. 1. Purification of the extracellular protease NS70 from *B. licheniformis*.

(A) Chromatography of crude enzyme on DEAE-Sepharose CL-6B column. The enzyme was eluted with a linear gradient of NaCl in 50 mM Tris-HCl buffer (pH 8.0), at a flow rate of 45 ml/h, and the volumn of each fraction was 5.6 ml. - ●-, $A_{280\ nm}$; - ○-, protease activity. (B) Phenyl Sepharose CL-4B chromatography of active fractions from CM-Sepharose CL-6B column. The enzyme was eluted with 10 mM phosphate buffer (pH 7.5), at a flow rate of 30 ml/h, and the volumn of each fraction was 3 ml. - ●-, $A_{280\ nm}$; - ○-, protease activity.

protease was purified using Phenyl-Sepharose column (Fig. 1B). A summary of the purification procedure is given in Table 3. Purification fold was 5.2 and 24% of enzyme activity was recovered. The purified enzyme showed a specific activity of 230 unit/mg protein when assayed at 55°C with Hammarsten casein as a substrate. The purified enzyme gave a single protein band on SDS-PAGE and its molecular weight was estimated to be 32,000 (Fig. 2).

Effects of Inhibitors, Metal Ions, and Detergents

After the enzyme was incubated with various inhibitors and metal ions at 30°C for 10 min in 50 mM Tris-HCl buffer (pH 8.0), and with each detergent at 30°C for 30 min in the same buffer, the remaining proteolytic activity of the enzyme was assayed. As shown in Table 4, the enzyme was markedly inhibited by 1 mM PMSF but not by EDTA and N-ethylmaleimide.

Table 3. Summary of purification steps of protease from thermophilic *Bacillus* sp. NS70.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture broth	80	3500	44	1.0	100
$(NH_4)_2SO_4$ 30-80%	36	2400	67	1.5	69
DEAE-Sepharose	8.7	1200	130	3.0	34
CM-Sepharose	7.4	1100	150	3.4	31
Phenyl-Sepharose	3.7	850	230	5.2	24

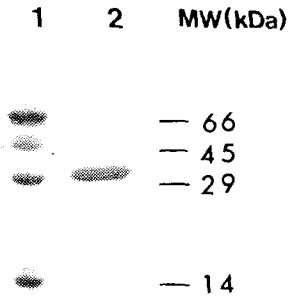

Fig. 2. SDS-PAGE of the purified protease NS70.
Lane 1: purified protease, *Lane 2:* molecular weight markers, bovine serum albumin (66 kDa), hen egg albumin (45 kDa), carbonic anhydrase (29 kDa) and lactalbumin (14.2 kDa).

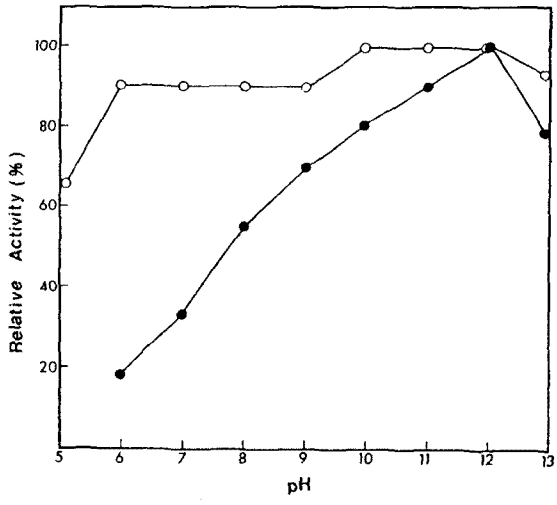

Fig. 3. Effects of pH on the protease activity and stability.
The enzyme activity was assayed at various pHs (●) and the enzyme stability was measured after incubation of the reaction mixture at various pHs for 1 h (○).

This result indicates that this enzyme is a serine protease. Cu^{2+} and Hg^{2+} ion inhibited enzyme activity at a final concentration of 2 mM, but K^+, Ca^{2+}, Mn^{2+} did not influence on the enzyme activity. This result is similar to the case of *Thermoactinomyces* sp. HS 682 (10). Detergent such as SDS did not affect proteolytic activity at 1% (w/v). The effect of inhibitors on enzyme activity was similiar to the case of subtilisin Carlsberg (9), Thermitase (2).

Table 4. Effect of inhibitors, metal ions, and detergents on the proteolytic activity.

Reagents	Concentration (mM)	Relative activity (%)
None		100
PMSF	1	4
EDTA	1	70
L-cysteine	1	65
KCN	1	112
N-etylmaleimide	1	126
KCl	2	99
$HgCl_2$	2	18
$MgCl_2$	2	85
$CaCl_2$	2	92
$MnCl_2$	2	102
$CuCl_2$	2	9
$ZnCl_2$	1	76
SDS[a]	1	92
Tween-80[b]	1	172
DTT[a]	1	134
β-mercaptoethanol[b]	1	87

[a]Their concentration units were % (w/v). [b]Their concentration units were % (v/v).

Effects of pH on Proteolytic Activity and Stability

The enzyme was stable at a broad pH range of pH 6-13 (Fig. 3). Subtilisin Carsberg has good activity for hydrolysis of urea-denatured haemoglobin between pH 6 and 11 (1). Its optimum pH for the proteolytic activity was pH 12 when Hammarsten casein was used as a substrate. This high optimum pH value was comparable with the optimum pHs of the proteases produced by some alkalophilic bacteria *Bacillus* sp. AH-101 (17), *Bacillus* sp. B18 (13), and *Thermoactinomyces* sp. HS 682 (10).

Effects of Temperature on Proteolytic Activity and Stability

The effect of temperature on the proteolytic activity of protease NS70 was examined at various temperatures varying from 20°C to 70°C. As shown in Fig. 4, the enzyme showed maximum activity at 65°C. The ther-

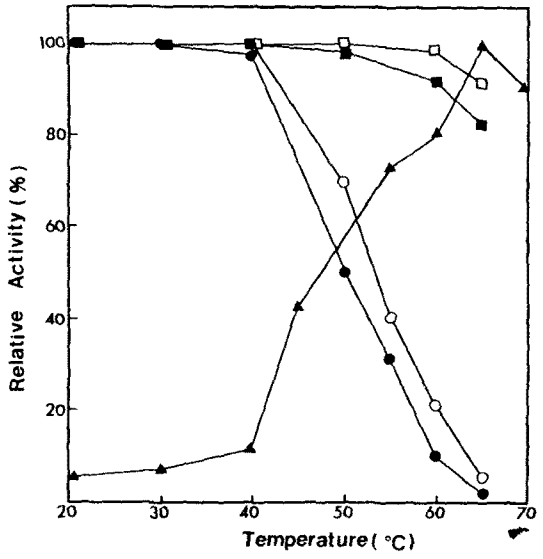

Fig. 4. Effects of temperature on the protease activity and stability.

The enzyme activity was assayed at various temperatures (▲) and the enzyme stability was measured using both crude and purified enzyme after incubation at various temperatures for 10 min with and without addition of 10 mM $CaCl_2$. - ●-, purified protease without $CaCl_2$; - ○-, purified protease with 10 mM $CaCl_2$; - ■-, crude protease without $CaCl_2$; - □-, crude protease with 10 mM $CaCl_2$.

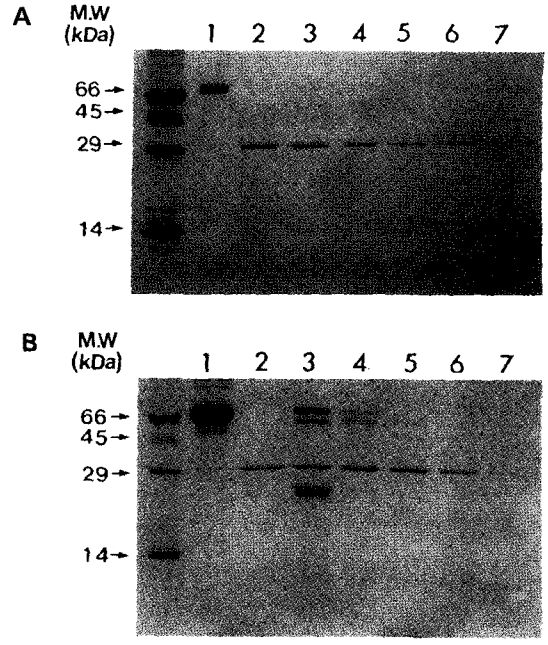

Fig. 5. The effect of temperature on autoproteolytic activity of the purified protease NS70.

One microgram of enzyme was incubated in the absence of BSA (A) or in the presence of 10 μg of BSA (B) at 30°C (lane 3), 40°C (lane 4), 50°C (lane 5), 60°C (lane 6), 65°C (lane 7) for 10 min. The reaction was stopped by adding phenylmethylsulfonyl fluoride (final concentration, 2 mM) and loaded on 12% SDS-polyacrylamide gel. Five micrograms (panel A, lane 1) or 10 μg (panel B, lane 1) of bovine serum albumin and 1 μg of the enzyme, which was heated at 100°C with PMSF were loaded as controls (lane 2).

mostability of purified protease NS70 was drastically decreased above 40°C. This was different from the melting point (Tm) of 68°C obtained by the fluorescence emission study. In order to test possible autolysis during the thermal inactivation of protease NS70, thermostability was examined at different temperatures (30-65°C) using purified and crude enzymes. The crude enzyme was stable up to 65°C, while the purified enzyme was stable below 45°C and showed remaining activity of only 5% at 65°C. This indicates that purified protease NS70 is degraded by autolysis at elevated temperatures and thereby inactivated. Also autolysis of purified protease NS70 was observed on SDS-polyacrylamide gel. As shown in Fig. 5A, the enzyme band disappeared above 50°C in the absence of bovine serum albumin by autoproteolytic digestion, which was correlated well with the thermostability profile of the purified enzyme in Fig. 4. In the presence of bovine serum albumin (Fig. 5B), the enzyme band was observed up to 60°C, which was also correlated well with the thermostability profile of the crude enzyme in Fig. 4.

To investigate whether or not Ca^{2+} ion has a stabilizing effect against both thermal denaturation and autoproteolytic degradation, fluorescence emission at 306 nm was measured at 35-80°C (Fig. 6). The decrease of fluorescence emission intensity in protein solution is due to the exposure of tyrosine residues in the hydrophobic

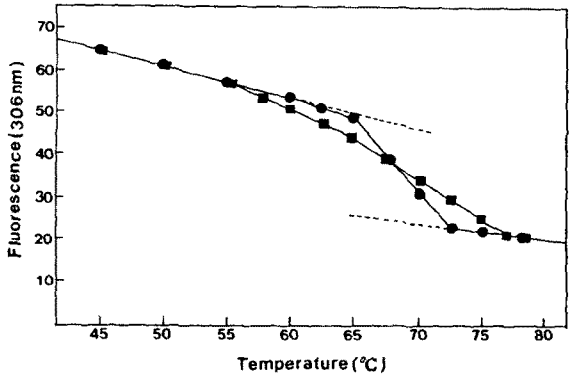

Fig. 6. Effects of temperature on the fluorescence emission of the protease NS70.

Fluorescence emission at 306 nm was measured with 275 nm excitation wavelength: - ●-, with 10 mM $CaCl_2$; - ■-, without $CaCl_2$.

environment to the polar aqueous solution. The fluorescence emission intensity decreased at 55°C in the absence of Ca^{2+}, while that point was elevated up to 65°C in the presence of 10 mM $CaCl_2$. This result was confirmed as shown in with Fig. 4, which shows that the re-

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          +1                              +14
DY        A-Q-T-V-P-Y-G-I-P-L-I-K-A-D
BPN       A-Q-S-V-P-Y-G-V-S-Q-I-K-A-P
CB        A-Q-T-V-P-Y-G-I-P-L-I-K-A-D
NS-70     A-Q-T-V-P-Y-G-I-P-L-I-K-A-D

          +164                                  +180
DY        T-I-G-Y-P-A-K-Y-D-S-V-I-A-V-G-A-V
BPN       T-V-G-Y-P-G-K-Y-P-S-V-I-A-V-G-A-V
CB        T-I-G-Y-P-A-K-Y-D-S-V-I-A-V-G-A-V
NS-70     T-I-G-Y-P-A-K-Y-D-S-V-I-A-V-G-A-D
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Fig. 7. The N-terminal amino acid sequences of the separating fragment of purified protease NS-70 from *B. licheniformis* and their comparison with those of subtilisin DY from *B. Subtilis* strain DY (DY), subtilisin BPN from *B. amyloliquefacience* (BPN), subtilisin Carlsberg from *B. licheniformis* (CB).

maining activity increased slightly at high temperature in the presence of 10 mM Ca^{2+}. This demonstrates that Ca^{2+} ion has a stabilizing effect against both autolysis and thermal denaturation of protease NS70.

N-Terminal Amino Acid Sequence Analysis

When the purified protease NS70 enzyme was kept at 4°C, two bands were observed with molecular weights of 15,000 and 17,000, respectively (data not shown). Therefore these bands were supposed to be fragments resulting from autoproteolytic action. As shown in Fig. 7, the N-terminal fourteen amino acid sequence of the large fragment was exactly the same as that of subtilisin type protease, subtilisin Carlsberg as previously reported (9). On the other hand, the seventeen amino acid sequence of the small fragment was also similar to that starting from the 164th amino acid of subtilisin Carlsberg. This indicates that autoproteolytic action of protease NS70 is not random cleavage but site-specific cleavage. Molecular size and N-terminal amino acid sequences of protease NS70 were similar to those of subtilisin Carlsberg from *B. licheniformis*, but some biochemical characteristics, especially pH profiles, of protease NS70 were different from those of Carlsberg, indicating that this enzyme is another subtilisin-type protease produced from thermophiles. We are now in the progress of cloning and sequencing the structural gene encoding this enzyme. We hope that this investigation will provide more information on the relationship between structure and function and hence make it possible to elucidate the difference in optimum pH between subtilisin Carlsberg and protease NS70 and enhance the thermostability of ordinary enzymes by amino acid substitutions using site-directed mutagenesis.

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