Journal of Microbiology and Biotechnology
Vol. 4, No. 2, 113-118, 1994

Purification and Characterization of a Thermostable Protease from *Pseudomonas aeruginosa* NS-83

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A bacterial strain NS-83 isolated from soil was able to produce an extracellular thermostable protease. The strain was identified as *Pseudomonas aeruginosa* based on its morphological and physiological characteristics. A thermostable protease from this strain has been purified to homogeneity as judged by SDS-PAGE and isoelectric focusing. The purification procedures included hydrophobic interaction, ion exchange, and gel filtration chromatography. The M_r and the pI of the enzyme were 32,000 and 5.9, respectively. The optimal pH at 55°C and the optimal temperature at pH 7.0 were 8.0 and 60°C, respectively. The D-values of the enzyme at 60, 65, and 70°C were 22, 2.1, and 0.75 hrs, respectively. The enzyme activity was significantly inhibited in the presence of 1 mM o-phenanthroline or EDTA, suggesting that the enzyme is metalloprotease. The K_m and V_{max} for Hammarsten casein were found to be 3.2 mg/ml and 0.918 unit/ml, respectively. These enzymatic properties were similar to those of elastase produced from *P. aeruginosa* IFO 3455, but the enzyme was clearly different from the reported elastase, in respect to Ca^{++} effects on enzyme-thermostability. This property, together with amino acid composition analysis, confirmed that the enzyme differs from the known *P. aeruginosa* elastase.

Proteases produced from microorganisms are widely used in the leather industry, pharmaceutical industry, food industry and waste processing. Proteases are probably the most important class of industrial enzymes with world-wide use (7). Until recently, major concern has been focused on screening proteases with a criterion set only as to higher enzyme activity. There are, however, increasing number of reports on protease having specific properties such as substrate specificity, regioselectivity, chiral selectivity, thermostability and solvent stability (16).

Thermostable proteases attract much attention because they have many advantages in biotechnological uses. Proteolysis of substrates can proceed more rapidly at higher temperatures by using thermostable proteases, and thermostable proteases can prevent the microbial contamination in food processing. In addition, the stability of thermostable proteases is not restricted to temperature but also include resistance to denaturing reagents, detergents and organic solvents (2, 3, 12, 15). In the presence of organic solvents, the ability of proteases to synthesize peptide bonds is enhanced (6). Therefore thermostable proteases can be used as biocatalysts in organic synthesis of useful compounds where enhanced solvent resistance is required.

We isolated a bacterial strain capable of producing an extracellular thermostable protease from soil, purified the thermostable protease, and examined its enzymatic characteristics in this research.

MATERIALS AND METHODS

Screening of Proteolytic Bacterium

Soil samples were suspended in sterile water and spread on a "Skim milk plate" containing modified Y medium (1% Polypeptone, 0.5% yeast extract, 0.2% beef extract, 0.2% glycerol, 0.3% NaCl, 0.2% KH_2PO_4, 0.2% K_2HPO_4, and 0.01% $MgSO_4$, pH 7.2), 1% skim milk (or 1% defatted soybean in a "Defatted soybean plate"), and 1.5% agar. The microorganisms showing a clear zone around a colony after incubation for 24 hrs at 37°C, were isolated. Among these a bacterial strain producing thermostable protease was selected.

Cultivation of *Pseudomonas aeruginosa* NS-83

P. aeruginosa NS-83 was cultivated in a 5 liter jar fer-

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Key words: protease, thermostability, *Pseudomonas aeruginosa*, elastase

mentor using 3 liters of modified Y medium at 37°C with an agitation rate of 400 rpm and an aeration rate of 0.5 vvm.

Assay of Protease Activity

Protease activity was assayed at 55°C with Hammarsten casein as a substrate (14). Three milliliters of 0.6% (w/v) Hammarsten casein in a 10 mM potassium phosphate buffer (pH 7.0) was preincubated for 10 min at 55°C, and the reaction was started by the addition of 0.5 ml of enzyme solution. After 30 min the reaction was stopped by the addition of 3.2 ml of 0.44 M trichloroacetic acid. The mixture was filtered with Whatman No. 5 filter paper and the A_{275} of the filtrate was determined. One unit was defined as the amount of enzyme required to change optical density at 275 nm of 0.1 in 1 min at 55°C.

Determination of Protein Concentration

The protein concentration was measured by the optical density at 280nm or Bradford method using a protein assay kit (Bio-Rad Lab., Richmond, Calif, U.S.A.) with bovine serum albumin as the standard.

Purification Procedures

The extracellular protease released into the culture supernatant of *P. aeruginosa* NS-83 was purified at 4°C by the following procedure. The supernatant obtained by centrifuging a 3 liter culture broth was concentrated to 200 ml with an ultrafiltration kit (Bio-Recovery, Inc., New Jersey, U.S.A.). The concentrate was brought to 30% saturation with solid ammonium sulfate, and allowed to stand overnight. After the precipitate was discarded by centrifugation, the supernatant was put on a column (2.2×17 cm) of Phenyl Sepharose CL-4B previously eqilibrated with a 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 M ammonium sulfate. After the column was washed with the same buffer, the enzyme was eluted with a linear descending gradient from 0.5 M to 0 M of ammonium sulfate and followed by an isocratic elution with a 10 mM potassium phosphate buffer. The fractions having protease activity were collected and concentrated by ultrafiltration with an Amicon PM 10 membrane. The concentrated solution was put on a column (2.2~17 cm) of DEAE-Sepharose CL-6B previously equilibrated with a 10 mM potassium phosphate buffer (pH 7.0). After the column was washed with the same buffer, the enzyme was eluted with a linear gradient formed from the buffer containing 0 to 0.5 M of KCl. The active fractions were collected and concentrated. The concentrated soution was put on a Sephacryl S-200 column (2.0×95 cm) previously equilibrated with 150 mM NaCl, 50 mM potassium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer. The enzyme thus purified was concentrated to more than 10 mg/ml by ultrafiltration and stored at −20°C.

Polyacrylamide Gel Electrophoresis

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described by Laemmli (9) using 10% acrylamide in slab gels. Proteins were stained with Coomassie Brilliant Blue R-250.

pH Effect on Protease Activity

To determine pH stability of the enzyme, 50 μl of enzyme solution was added to 350 μl of 0.1 M sodium acetate (pH 4~6), potassium phosphate (pH 6~7.5), Tris-HCl (pH 7.5~9), and glycine (pH 9~11) buffers. After one hour-standing at room temperature, the remaining protease activity was assayed using 70 μl of each enzyme solution as described previously. To find the optimum pH of the enzyme, the protease activities at various pHs were checked.

Temperature Effect on Protease Activity

The optimum temperature of the enzyme activity was determined by assaying the protease activities at various temperatures as previously described. The temperature effect for the stability of the enzyme was tested as follows. Enzyme solutions were heat-treated at various temperatures for 30 min, centrifuged and the residual activities of the supernatants were assayed.

Fluorescence Spectroscopy

The experiment was done in a 20 mM Tris-HCl buffer (pH 7.3) containing 1 mM EDTA. Fluorescence spectra were measured on a Jasco FP 770 Spectrofluorometer in a thermostatted cuvette. Thermal unfolding was monitored by following the decrease in the fluorescence intensity at 303 nm (after excitation at 280 nm) with a heating rate of 1°C/min at a 2 μM protein concentration.

N-Terminal Amino Acid Sequencing

N-terminal amino acid sequencing of the enzyme preparation was carried out according to the modified method reported by Applied Biosystems Inc.. The sequence was determined by Edman degradation in an Applied Biosystems model 471A Protein/Peptide sequencer (Applied Biosystems Ins., CA, U.S.A.).

Amino Acid Composition Analysis

The enzyme was analyzed for amino acid composition after dissolving in 6 N HCl, sealed under nitrogen, and hydrolyzed for 24 hrs at 110°C. A Durrum D-500 amino acid analyzer was used.

RESULTS AND DISCUSSION

Isolation and Identification of Proteolytic Bacterium

Bacterial strains capable of producing extracellular protease were isolated from soil samples collected at several sites in Korea. Among these, isolate NS-83 sho-

Table 1. Microbiological characteristics of the isolate NS-83

Gram stain	−
Rods	
Width (μ)	0.43
Length (μ)	1.2
Motility	+
Growth at 41°C	+
Enzyme activity of	
Catalase	+
Arginine dihydrolase	+
Formation of indole	−
Denitrification	+
Gelatin liquefaction	+
Carbon sources for growth	
Glucose	+
Mannitol	+
2-ketogluconate	+
Inositol	−
L-Alanine	+
L-Serine	−

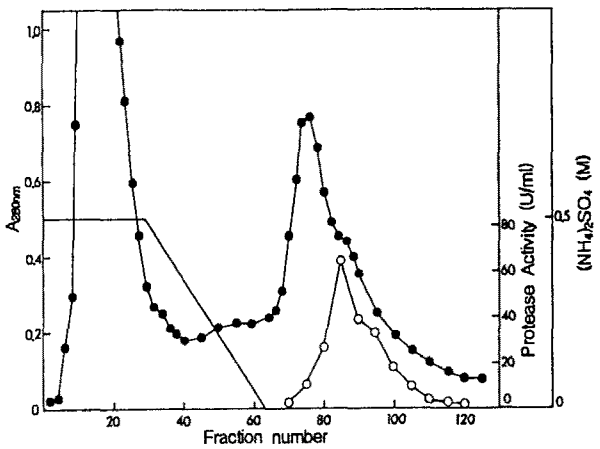

Fig. 1. Chromatography of crude enzyme on Phenyl Sepharose CL-4B column.
The enzyme was eluted with a decreasing gradient of phosphate buffer containing ammonium sulfate, at a flow rate of 50 ml/h, and the volume of each fraction was 10 ml. −●−; $A_{280\,nm}$, −○−; protease activity.

wed the highest thermostable proteolytic activity against skim milk and defatted soybean when assayed at 55°C. Identification of the strain was carried out according to the method in Bergey's Manual (8). The microbiological and biochemical characteristics of isolate NS-83 were shown in Table 1. The isolate was motile, Gram-negative, and rod-shaped. Cells were 0.43 μm wide and 1.2 μm long when measured under a scanning electron microscope. The isolate showed high catalase and arginine dihydrolase activities. These results indicated that isolate NS-83 be a strain of *Pseudomonas aeruginosa*. So isolate NS-83 was named *Pseudomonas aeruginosa* NS-83.

Purification of the Protease

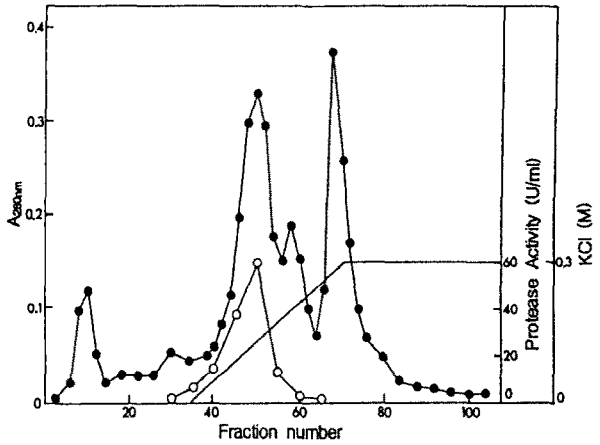

Fig. 2. DEAE-Sepharose CL-6B chromatography of active fractions from Phenyl Sepharose CL-4B column.
The enzyme was eluted with a linear gradient of KCl in a phosphate buffer, at a flow rate of 50 ml/h, and the volume of each fraction was 10 ml. −●−; $A_{280\,nm}$, −○−; protease activity.

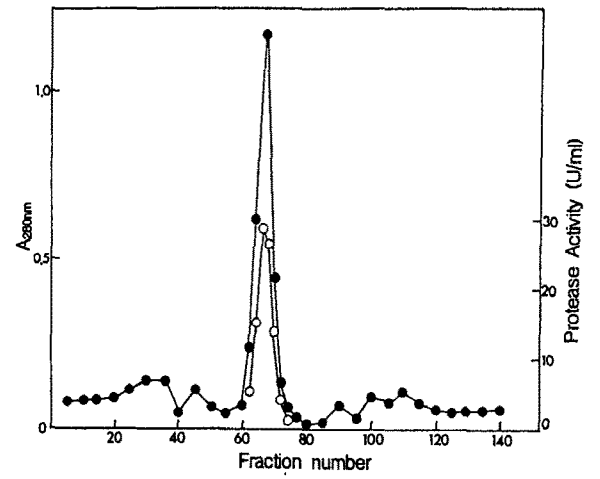

Fig. 3. Sephacryl S-200 gel filtration chromatography of active fractions from DEAE-Sepharose CL-6B column.
The enzyme was eluted with a phosphate buffer, at a flow rate of 40 ml/h, and the volume of each fraction was 2 ml. −●−; $A_{280\,nm}$, −○−; protease activity.

The purification of the enzyme was done as described in Materials and Methods. The results of Phenyl-Sepharose CL-4B, DEAE-Sepharose CL-6B, and Sephacryl S-200 column chromatography were shown in Fig. 1, 2, and 3. The purified enzyme was homogeneous as judged by SDS-PAGE (Fig. 4) and isoelectric focusing, and the molecular weight and the pI were estimated to be 32,000 and 5.9, respectively. The typical results of the purification procedure were summarized in Table 2. The purified enzyme had a specific activity of 175 units/mg protein when assayed at 55°C with Hammarsten casein as a substrate.

Biochemical and Enzymatic Characteristics of the Protease

The biochemical and structural characteristics of the

protease were studied with the purified enzyme. The protease activities were measured from pH 4.0 to 11.0 using various buffer solutions described in Materials and Methods. The enzyme had an optimum pH of 8.0 as shown in Fig. 5 and it was fairly stable at pH values ranging from 5.0 to 10.0.

The proteolytic activities were measured at various temperatures ranging from 20°C to 80°C. The optimum temperature was found to be 60°C as shown in Fig. 5. The thermostability of the enzyme was measured after incubation for predetermined periods ranging from 5 to 30 min at each temperature. The residual activities as a function of heat treatment time were shown in Fig. 6. Heating of the enzyme resulted in first-order inactivation kinetics, showing that the D-values at 60, 65 and 70°C were 22, 2.1 and 0.75 hrs, respectively. There have been a few reports on thermostable proteases from some mesophilic bacteria (5). The protease from *P. aeruginosa* NS-83 seemed to be more thermostable than subtilisin Carlsberg and BPN from *Bacillus subtilis*, whose half lives of enzymatic activity were reported to be 0.5 min and 0.1 min at 60°C, respectively, and its thermostability was comparable to the elastase from *P. aeruginosa* which was known to have residual activity of 71% after incubation for 10 min at 65°C (11).

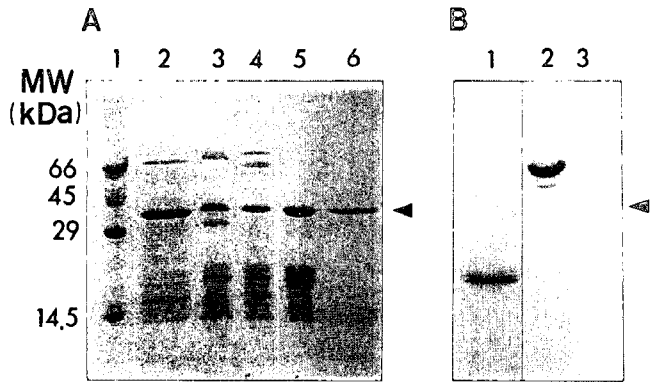

Fig. 4. SDS-PAGE (A) and isoeletric focusing (B) of the protease.
(A), Lane 1; molecular weight markers, lane 2; culture supernatant, lane 3; after ammonium sulfate precipitation, lane 4; after Phenyl Sepharose CL-4B chromatography, lane 5; after DEAE-Sepharose CL-6B chromatography, lane 6; after Sephacryl S-200 gel filtration. (B), Lane 1; trypsin inhibitor from soybean (pI 4.6), lane 2; human carbonic anhydrase (pI 6.6), lane 3; the purified protease.

The temperature dependence of fluorescence emission properties of the enzyme molecules was shown in Fig. 7. The fluorescence emission intensity slightly decreased upon heating up to 54°C, while a rather drastic transition in fluorescence emission intensity was observed in the broad range from 54 to 82°C with a T_m

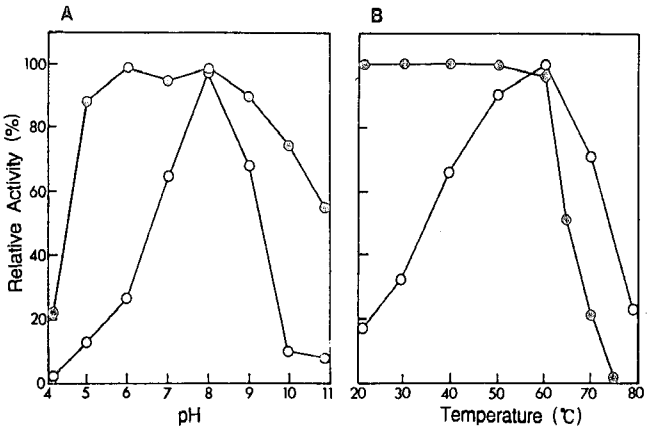

Fig. 5. Effects of pH (A) and temperature (B) on enzyme activity and stability.
The enzyme was first assayed at various pHs (○; A) and temperatures (○; B). Next, the enzyme was assayed again after incubation of the reaction mixture at various pHs (●; A) for 1 hr and temperatures (●; B) for 30 min.

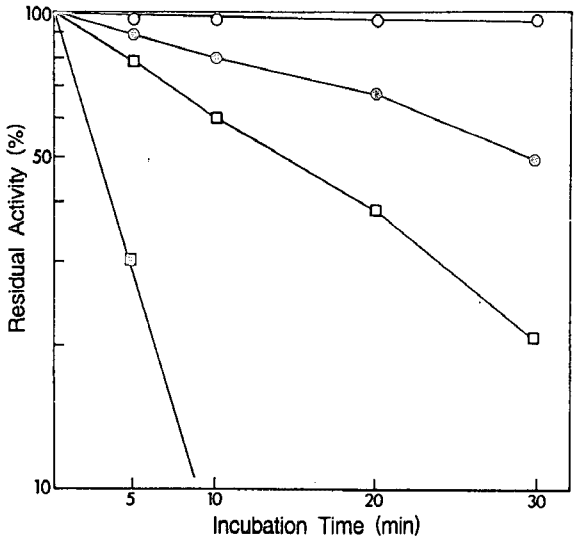

Fig. 6. Thermostability of the protease.
The enzyme solutions were incubated at 60 (○), 65 (●), 70 (□), and 75 (■) °C. The residual activities of heat treated enzymes were measured at 55°C.

Table 2. Summary of purification of protease from *P. aeruginosa* NS-83

Steps	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
Culture Sup.	1710	94,500	55.3	1	100
$(NH_4)_2SO_4$	1440	66,600	46.3	0.84	70.5
Phenyl-Sepharose	675	34,500	51.1	0.92	36.5
DEAE-Sepharose	333	23,100	69.4	1.3	24.4
Sephacryl S-200	108	18,900	175	3.2	20.0

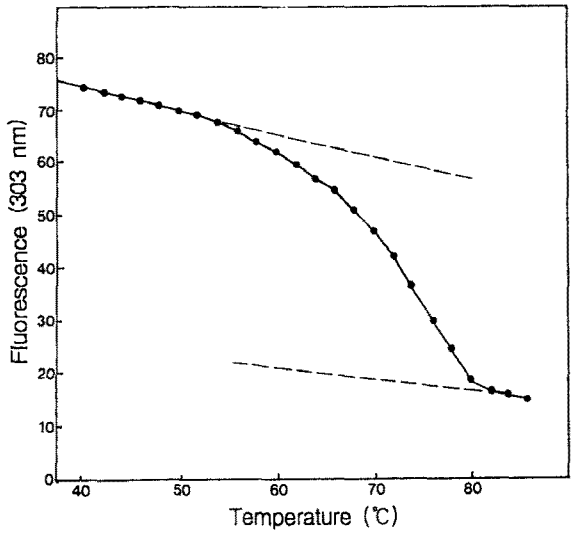

Fig. 7. Effects of temperature on the fluorescence emission of the protease.
Fluorescence intensity at 303 nm was measured with a 280 nm excitation wavelength.

Table 3. Effects of inhibitors and metal ions on proteolytic activity

Reagents	Relative activity (%)	
	1 mM	5 mM
PMSF	88	75
Aprotinin	100	
EDTA	27	20
o-Phenanthroline	4.3	2.5
KCN	99	74
L-Cysteine	20	23
$CuCl_2$	30	28
$CaCl_2$	100	42
$MnCl_2$	71	29
$MgCl_2$	110	88
$HgCl_2$	20	1.9
$ZnCl_2$	29	20

of 73°C. From the slow slope, it was supposed to have had some intramolecular disulfide bonds which may help to stabilize the tertiary structure of the protein. The high T_m value and relatively slow unfolding of the protein structure correlated well with the high level of resistance against heat inactivation of enzyme activity.

The purified enzyme was preincubated with various reagents of 1 mM and 5 mM concentrations for 10 min at 37°C and the enzyme activities were assayed. As shown in Table 3, serine protease inhibitors did not affect the enzyme activity, whereas both EDTA and o-phenanthroline strongly inhibited the enzyme activity, which suggested that it is a metalloprotease. Among heavy metals, Hg^{++} and Zn^{++} almost completely inhibited the proteolytic activity of the enzyme at 5 mM

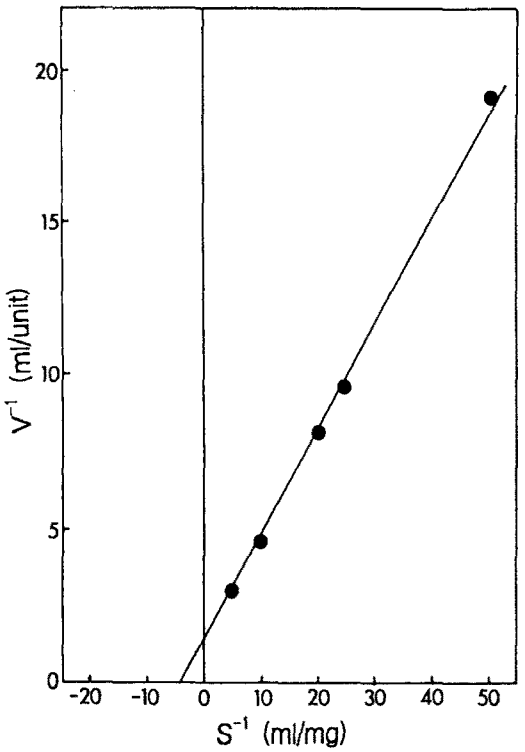

Fig. 8. Lineweaver-Burk plot for the hydrolysis of Hammarsten casein by the purified protease.

Table 4. N-terminal sequence of *P. aeruginosa* NS-83 protease

Enzyme	N-terminal sequence
P. aeruginosa NS-83 protease	N-Ala-Glu-Ala-Gly-Gly-Pro-Gly-Gly-Asn-Gln-Lys-Ile-Gly-Lys-Tyr-Thr-Tyr-Gly-

concentration.

The K_m and V_{max} of the enzyme for Hammarsten casein were estimated to be 3.2 mg/ml and 0.918 unit/ml, respectively (Fig. 8).

P. aeruginosa has been reported to produce three kinds of proteases, that is, elastase, alkaline protease and Las A protein (10, 11, 13). The purified enzyme showed several biochemical and enzymatic characteristics similar to those of elastase and the N-terminal eighteen amino acids of the purified enzyme (Table 4) were identical with those of the elastase. However, the enzyme was different from elastase in that the thermostability of the enzyme was not increased by Ca^{++} ions ranging from 1 to 30 mM concentration (unpublished data), whereas elastase has four Ca^{++} binding sites and its thermostability was highly increased by Ca^{++} addition at the similar concentration range (4). In addition, the amino acid composition of two proteases showed some differences (Table 5). Recently, the structural genes encoding the elastase were cloned from two *P. aeruginosa* strains (1, 4). The mature elastases from both strains

Table 5. Amino acid composition of *P. aeruginosa* NS-83 protease and elastase

Amino acid	Number of residues	
	P. aeruginosa NS-83 protease	elastase
Asx	45	42
Thr	18	18
Ser	24	25
Glx	24	16
Pro	12	10
Gly	39	33
Ala	33	27
Cys	ND*	4
Val	21	19
Met	3	9
Ile	12	9
Leu	18	14
Tyr	6	22
Phe	15	16
His	6	7
Lys	9	11
Arg	12	15
Trp	ND	4

* ND; not determined

were preceded by a signal sequence and a large propeptide and differed only in one residue. The purified enzyme under this study might be another kind of protease with some sequence difference from the two reported elastases.

We are currently studying the detailed physicochemical and enzymological properties of the thermostable protease from *P. aeruginosa* NS-83 as well as its gene cloning. These results will be reported shortly.

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(Received March 31, 1994)