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Affinity Chromatography of Alkaline Proteinase S on *N*-Carbobenzoxyglycylleucylaminohexyl-Sepharose

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Alkaline proteinase produced by one of the monospore isolates of *Streptomyces violaceorectus* MC675-A8, a producer of the alkaline metalloendopeptidases named alkinonases A and AF, was purified by affinity chromatography on *N*-benzyloxycarbonylglycylleucylaminohexyl-Sepharose to electrophoretic homogeneity.

The enzyme was inactivated by phenylmethanesulfonyl fluoride and diisopropylfluorophosphate, but not by chelating agents or sulfhydryl reagents, and was distinct from alkinonases A and AF. The optimum pH for casein hydrolysis was 9.5—10.5, and the enzyme was stable within a pH range of 5.0—12.0. The molecular weight was estimated to be 30000. The peptidase activity of the enzyme was greatest on *N*-benzyloxycarbonylglycylprolylleucylglycine ethyl ester among the synthetic substrates tested in this work. Amidase activity was observed towards peptide amides such as *N*-benzyloxycarbonylglycylleucine amide and *N*-benzyloxycarbonylglycylphenylalanine amide. The enzyme showed anti-inflammatory activity against carrageenan-induced edema in rats, as did alkinonases A and AF. Bradykinin was hydrolyzed by the enzyme to arginylprolylprolylglycylphenylalanylserylprolylphenylalanine and arginine.

Since the enzyme showed a serine proteinase nature, the enzyme was named alkaline proteinase S, and the producing microbe was designated as *Streptomyces violaceorectus* MC675-A8-S.

Keywords——alkaline proteinase S; affinity chromatography; serine proteinase; *N*-benzyloxycarbonylglycylleucylaminohexyl-Sepharose; *Streptomyces violaceorectus*; bradykinin

Alkinonases A and AF were obtained from the culture broth of *Streptomyces violaceorectus* MC675-A8 as anti-bradykinin substances by Nakamura *et al.*[1] These enzymes are alkaline metalloendopeptidases with potent anti-inflammatory activity against carrageenan-induced edema in rats.[1] A marked decrease in the enzyme-producing activity of this strain was, however, observed during maintenance in agar slant cultures.

One of the monospore isolates of this strain was found to release large amounts of the proteinases (1000—2500 units/ml as casein hydrolysis activity at pH 9.0), 2 to 5 times more than the amount originally reported.[2] The main component was purified to electrophoretic homogeneity by affinity chromatography on *N*-benzyloxycarbonylglycylleucylaminohexyl-Sepharose, which had been used in the purification of alkinonase A.[2] The enzyme showed different behavior on this adsorbent from that of alkinonase A. Because of the serine proteinase nature of the enzyme, it was named alkaline proteinase S.

The details of the purification and characterization of alkaline proteinase S are presented in this paper.

Materials and Methods

Materials——*N*-Benzyloxycarbonyl peptides such as Z–Gly–Leu, Z–Gly–Leu–NH_2, Z–Gly–Pro–Leu–Gly and

Z–Gly–Pro–Leu–Gly–Pro were products of the Peptide Institue, Protein Research Foundation, Bradykinin was purchased from Sigma Chemicals Inc., and AH-Sepharose was obtained from Pharmacia Fine Chemicals Co. All other materials were commercial products of analytical grade and the amino acids were all of L-form.

Production of Alkaline Proteinase S——A seed inoculum was cultured in a 500 ml Sakaguchi flask containing 125 ml of a medium composed of 2.0% soluble starch, 2.0% glucose, 2.0% soybean meal, 0.5% yeast extract, 0.25% NaCl, 0.32% $CaCO_3$, 0.0005% $CuSO_4 \cdot 5H_2O$, 0.0005% $MnCl_2 \cdot 4H_2O$ and 0.005% $ZnSO_4 \cdot 7H_2O$ (pH 7.4, before sterilization) at 27 °C for 48 h on a reciprocal shaker (7 cm amplitude, 130 strokes/min). The seed culture (2 ml) was inoculated into a 500 ml Sakaguchi flask containing 125 ml of a producing medium consisting of 2.0% soluble starch, 2.0% glucose, 0.5% yeast extract, 0.2% L-asparagine·H_2O, 0.25% NaCl, 0.64% $CaCO_3$, 0.005% $CuSO_4 \cdot 5H_2O$, 0.0005% $MnCl_2 \cdot 4H_2O$ and 0.005% $ZnSO_4 \cdot 7H_2O$ (pH 7.4). The culture was conducted at 27 °C for 6 d on the reciprocal shaker.

Isolation of Crude Enzyme——The culture broth filtrate (1780 ml) was brought to 85% saturation with ammonium sulfate and the pH was adjusted to 7.0 by the addition of 1 N NH_4OH. The precipitate was collected by centrifugation, redissolved in a small volume of H_2O (200 ml), dialyzed against distilled H_2O containing 1 mM $Ca(OAc)_2$ and precipitated with cold acetone to give crude enzyme (631.9 mg) with 93% recovery of the caseinolytic activity. The crude enzyme was passed through a DEAE-cellulose column to remove melanin-like contaminants prior to affinity chromatography.

Preparation of Z–Gly–Leu–AH–Sepharose——The details were described previously.[2] Briefly, a mixture of AH-Sepharose (20 ml as H_2O-swelled gel/5 mg as dry weight), Z–Gly–Leu (150 mg in 20 ml of 40% aqueous *N*, *N*-dimethylformamide, adjusted to pH 5.0) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (456 mg) was left at room temperature overnight with gentle shaking.

Assay for Protein Determination——The protein concentration was measured by the method of Lowry *et al.*[3] using bovine serum albumin as a standard.

Assay of Caseinolytic Activity——Proteolytic activity against casein was measured by the casein-275 nm method of Hagihara *et al.*[4] with some modification as described in the previous paper.[1] One unit of the caseinolytic activity was defined as the amount of enzyme producing an absorbance equivalent to 1 μg of tyrosine per min at 37 °C.

Hydrolysis of Synthetic Substrates——The hydrolysis retes of various synthetic substrates were determined as follows. A mixture of an aqueous enzyme solution (250 μl) and 5 mM substrate solution (250 μl) in 50 mM Tris-HCl buffer (pH 9.0) was incubated at 37 °C for 10 min. After the addition of 0.1 N AcOH (250 μl) and then Moore's ninhydrin solution[5] (500 μl), the whole was kept at 100 °C for 10 min. The absorbance at 570 nm was measured against a blank to determine the hydrolysis rate, with glycine as a standard.

The scissile bond of the peptide was identified by silica gel thin layer chromatography (TLC). The reaction mixture [composed of aqueous enzyme solution (500 μl) and 5 mM substrate solution (500 μl) in 10 mM Tris-HCl buffer (pH 9.0)] was lyophilized after a 2 h incubation at 37 °C. The residue was dissolved in MeOH (100 μl) and charged on a silica gel thin layer plate (Merck, Kieselgel 60 F_{254}) which was developed with either *n*-BuOH–AcOH–H_2O (4 : 2 : 1) or iso-PrOH–AcOEt–NH_4OH (7 : 9 : 4). The digested substrate was analyzed on thin layer plates with detection by ultraviolet (UV) irradiation and by spraying 1% ninhydrine solution.

Hydrolysis of Bradykinin by Alkaline Proteinase S——A mixture of aqueous bradykinin solution (0.3 mg/600 μl), 1/15 M phosphate buffer (20 μl, pH 9.0) and aqueous enzyme solution (13 μg/20 μl) was incubated at 37 °C for 5 h, lyophilized, redissolved in 0.1 M $NaHCO_3$ (200 μl) and reacted with dansyl chloride (0.5 mg/200 μl of acetone). Separation of dansyl peptides and determination of N-terminal amino acids of the peptides were done according to the methods previously reported.[6]

Electrophoresis——Electrophoresis was performed in 10% polyacrylamide gel in the presence of sodium dodecyl sulfate according to the the method of Weber and Osborn.[7] Bovine serum albumin (MW 68000), α-chymotrypsinogen (MW 25700) and lysozyme (MW 14300) were used as reference proteins to determine the molecular weight of the purified enzyme.

Anti-inflammatory Activity——Male Slc-Wistar rats (120—135 g) were employed. At 30 min after intraperitoneal administration of the enzyme solution, 0.1 ml of 2% carrageenan in 0.9% NaCl was injected subcutaneously into one hind paw of each of the 5 rats. The volume of foot swelling was measured in terms of fluid displacement at 1, 3 and 5 h after the injection of carrageenan.

Results

Purification of Alkaline Proteinase S

An acetone powder (105 mg) was dissolved in 10 ml of 1/15 M phosphate buffer (pH 7.5) and charged onto a DEAE-cellulose column (6.0 cm × 1.6 cm, i.d.) equilibrated with the same buffer. The active eluate from the DEAE-cellulose column was adsorbed on a Z–Gly–Leu–AH–Sepharose column (10.0 cm × 1.0 cm, i.d.) from 1/15 M phosphate buffer (pH 7.5). The

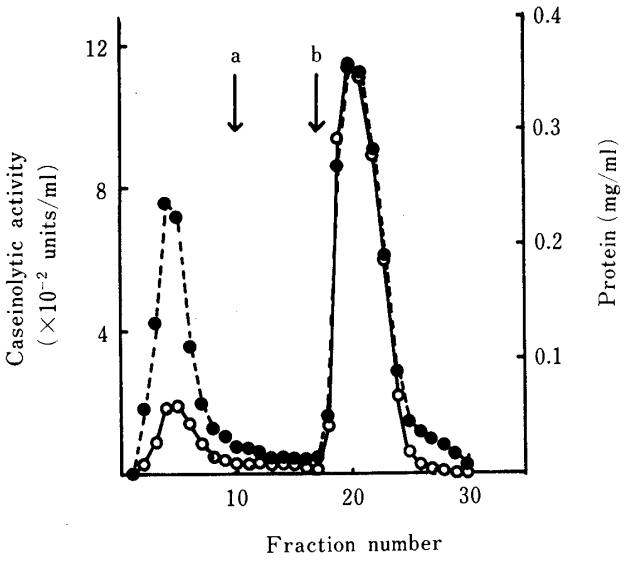

Fig. 1. Affinity Chromatography of Alkaline Proteinase S on Z–Gly–Leu–AH–Sepharose

The crude enzyme (10 mg as protein) after treatment with DEAE-cellulose in 1/15 M phosphate buffer (pH 7.5) was charged on a Z–Gly–Leu–AH–Sepharose column (10.0 cm × 1.0 cm, i.d.). Arrow a indicates change of the buffer from 1/15 M phosphate buffer (pH 7.5) to 1/15 M phosphate buffer (pH 7.5) containing 5 mM EDTA and arrow b indicates further change to 1/15 M phosphate buffer (pH 5.5). The eluate was collected in 4.2 ml fractions.

○, caseinolytic activity; ●, protein concentration.

TABLE I. Purification of Alkaline Proteinase S

Enzyme fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
Acetone powder (105 mg)	68.6	129000	1880	100
DEAE-cellulose chromatography	53.1	129000	2430	100
Z–Gly–Leu–AH–Sepharose affinity chromatography	24.5	97000	3970	75

column was throughly washed with the same buffer containing 5 mM ethylenediamine tetraacetic acid (EDTA), then the enzyme was eluted from the column with 1/15 M phosphate buffer (pH 5.5) with 75% recovery of the original activity as shown in Fig. 1. The purification of alkaline proteinase S is summarized in Table I.

Homogeneity and Molecular Weight Estimation

The purified enzyme showed a single band on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as shown in Fig. 2 and was eluted as a single peak on Sephadex G-75 gel filtration. The molecular weight of the purified enzyme was estimated to be 30000 by SDS-polyacrylamide gel electrophoresis and Sephadex G-75 gel filtration.

Effect of pH on Caseinolytic Activity and Stability at Various pH Values

The optimum hydrolysis of casein by alkaline proteinase S was observed in the region of pH 9.5—10.5 (Fig. 3). The enzyme was stable at pH 5.0—12.0 as shown in Fig. 4.

Peptidase and Amidase Activities of Akaline Proteinase S

The hydrolytic activity of the enzyme on synthetic substrates was examined and the results are presented in Table II. Peptidase activity was measured by Moor's ninhydrin method.[5] *N*-Benzyloxycarbonylglycylprolylleucylglycine ethyl ester was the best substrate among those tested here, followed by *N*-benzyloxycarbonylglycylprolylleucylglycylproline and *N*-benzyloxycarbonylglycylprolylleucylglycine in that order. The scissile bonds, indicated by arrows in the table, were identified by TLC of the hydrolysates. Amidase activity was demonstrated on amide type compounds such as *N*-benzyloxycarbonylglycylleucine amide and *N*-benzyloxycarbonylglycylphenylalanine amide, but not on glycylphenylalanine amide.

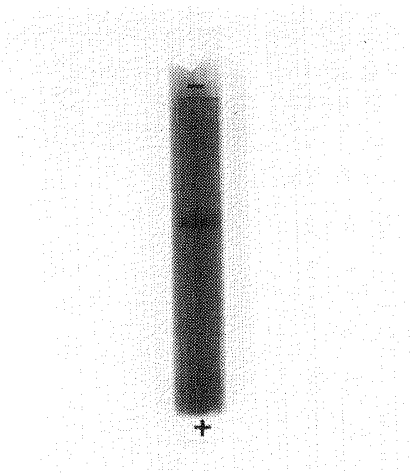

Fig. 2. SDS–Polyacrylamide Gel Electrophorogram of the Purified Enzyme

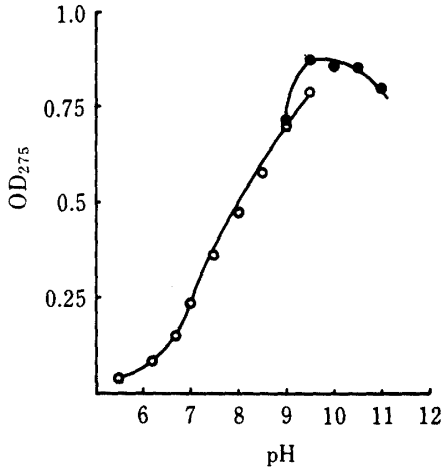

Fig. 3. Effect of pH on Caseinolytic Activity

A mixture of the aqueous enzyme solution (5.6 μg/ml) and 1% casein solution (1 ml) in 0.1 M Tris-HCl buffer (pH 5.5—9.5) or 0.1 M carbonate buffer (pH 9.0—11.0) was incubated at 37 °C for 20 min. ○, Tris-HCl buffer; ●, carbonate buffer.

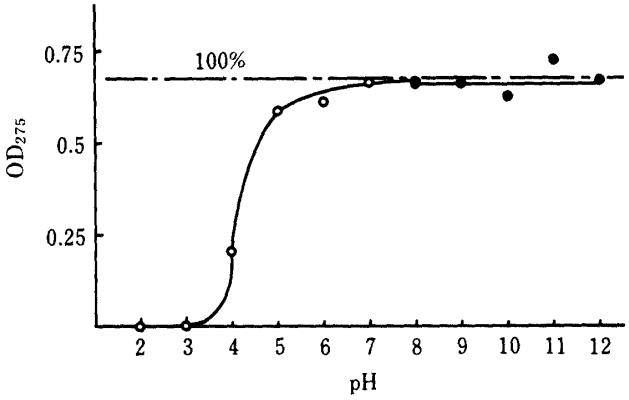

Fig. 4. pH Stability of Alkaline Proteinase S

A mixture of the aqueous enzyme solution (5.6 μg/0.1 ml) and 0.1 M buffer solution (0.1 ml) was left at room temperature for 1 h. The pH was adjusted to 9.0 and the total volume to 1.0 ml, then the enzyme solution was incubated with 1% casein solution (1 ml) in 0.1 M Tris-HCl buffer (pH 9.0) at 37 °C for 20 min. ○, phosphate-citrate buffer (pH 2.0—8.0); ●, glycine–NaOH buffer (pH 8.0—12.0).

N-Benzyloxycarbonylglycylleucine and *N*-benzyloxycarbonylglycylphenylalanine, which are specific substrates for carboxypeptidase, were not hydrolyzed. The hydrolysis rate of Z–Gly–Pro–Leu–Gly was reduced in the presence of Z–Gly–Leu–NH_2 as shown in Table III.

Effect of Various Chemicals

As shown in Table II, the enzyme showed carboxypeptidase-like activity on the synthetic substrate Z–Gly–Pro–Leu–Gly, as well as endopeptidase activity. The effects of various chemicals on the caseinolytic activity and the hydrolysis rate of Z–Gly–Pro–Leu–Gly were compared, and the results are shown in Table IV. Both activities were inactivated by phenylmethanesulfonyl fluoride and diisopropylfluorophosphate, but not by chelating agents or sulfhydryl reagents.

Anti-inflammatory Activity

The anti-inflammatory activity of alkaline proteinase S against carrageenan-induced edema in rats was determined by intraperitoneal injection. Alkaline proteinase S had dose-dependent anti-inflammatory activity as shown in Table V. ID_{30} of alkaline proteinase S against carrageenan-induced edema was 0.25 mg/kg of body weight at 3 h after the injection of carrageenan. In autopsy after the anti-inflammatory test, it was found that alkaline proteinase S at the doses of 3.0 and 5.0 mg/kg induced peritoneal irritation, but no irritation was observed at doses below 1.0 mg/kg.

TABLE II. Hydrolysis of Synthetic Substrates

Amino acid or peptide	Hydrolysis I[a]	Hydrolysis II[b]	Amino acid or peptide	Hydrolysis I[a]	Hydrolysis II[b]
Z–Gly–Pro–Leu↓Gly–Pro	+	0.807[c]	Z–Gly–Phe	–	
Z–Gly–Pro–Leu↓Gly–OEt	+	2.135	Z–Glu–Phe	–	
Z–Gly–Pro–Leu↓Gly	+	0.193	Z–Phe–Tyr	–	0.004
Boc–Pro–Leu–Gly–NH_2	–	0.007	Z–Gly	–	
Z–Gly–Leu–Tyr	–		Z–Phe	–	
Z–Gly–Leu↓NH_2	+	0.001	ClAc–Tyr	–	
Z–Gly–Phe↓NH_2	+		Gly–Leu–Tyr	–	
Z–Pro–Leu↓NH_2	+		Gly–Leu	–	
Z–Trp–Leu↓NH_2	+		Gly–Phe	–	
Z–Gly–Leu	–	0.000	Gly–Phe–NH_2	–	

All amino acids were of L-form. Boc-, *tert*-Butoxycarbonyl; ClAc-, chloroacetyl.

a) Method I; A mixture of the aqueous enzyme solution (18.5 μg/500 μl) and 5 mM substrate solution (500 μl) in 10 mM Tris-HCl (pH 9.0) was incubated at 37 °C for 2 h. The reaction mixture was lyophilized, then the residue was dissolved in MeOH (100 μl) and subjected to silica gel thin layer chromatography developed with *n*-BuOH–AcOH–H_2O (4:2:1) or iso-PrOH–AcOEt–NH_4OH (7:9:4). The digested substrate was detected under a UV lamp, and then by spraying 1% ninhydrin solution. Arrows indicate the scissile bonds. +, hydrolyzed; –, not hydrolyzed.

b) Method II, A mixture of 5 mM substrate solution (250 μl) in 50 mM Tris-HCl (pH 9.0) and the aqueous enzyme solution (1.25, 5.0 or 12.5 μg/250 μl) was incubated at 37 °C for 10 min. After the addition of 0.1 N AcOH (250 μl) and Moor's ninhydrin solution (500 μl), the whole was kept at 100 °C for 10 min. The absorbance at 570 nm was measured against the blank. The hydrolysis rates were calculated using glycine as a standard based on MW 30000.

c) Hydrolysis rate (mol/mol enzyme/s).

TABLE III. Inhibition by Z–Gly–Leu–NH_2 of Hydrolysis of Z–Gly–Pro–Leu–Gly by Alkaline Proteinase S

Enzyme ($\times 10^6$ M)	Z–Gly–Pro–Leu–Gly (mM)	Z–Gly–Leu–NH_2 (mM)	Hydrolysis rate (mol/mol enz./s)	Inhibition (%)
9.17	1.67	0.00	0.202	0
9.17	1.67	1.67	0.081	60

A mixture of 5 mM Z–Gly–Pro–Leu–Gly solution (250 μl) in 25 mM Tris-HCl (pH 9.0), 5 mM Z–Gly–Leu–NH_2 solution (250 μl) in the same buffer and aqueous enzyme solution (20.6 μg/250 μl) was incubated at 37 °C for 10 min. After the addition of 0.1 N AcOH (250 μl) and then Moore's ninhydrin solution (500 μl), the whole was kept at 100 °C for 10 min. The released glycine was determined by measuring the absorbance at 570 nm against the blank. A molecular weight of 30000 was used for calculation of the hydrolysis rate of Z–Gly–Pro–Leu–Gly.

Hydrolysis of Bradykinin by Alkaline Proteinase S

The enzyme showed anti-inflammatory activity against carrageenan-induced edema in rats (Table V). Hydrolysis of bradykinin, one of the important mediators of inflammation, was analyzed according to the reported method.[6]

Bradykinin was hydrolyzed by alkaline proteinase S and the hydrolysate was dansylated as described in materials and methods. The resulting dansyl (DNS) peptides were separated into two bands (*Rf* values, 0.26 and 0.19) on a silica gel thin layer plate with iso-ProOH–AcOEt–28% NH_4OH (7:9:4). Each component was eluted from the silica gel with acetone–H_2O–pyridine–AcOH (50:50:1:3) and hydrolyzed with 6 N constant-boiling HCl in a sealed tube at 90 °C for 24 h. The N-termini of both components were identified as DNS-arginine by

TABLE IV. Effects of Various Chemicals on Casein Hydrolysis and Hydrolysis of Z–Gly–Pro–Leu–Gly

Chemicals	Residual activity (%) I[a]	II[b]
p-Chloromercuribenzoate	97	100
Monoiodoacetate	94	88
Glutathione (oxidized)	96	
Glutathione (reduced)	96	
L-Cysteine	96	
Ethylenediaminetetraacetate	104	95
8-Hydroxyquinoline	84	134
Iodine	9	
N-Bromosuccinimide	44	59
Sodium dodecyl sulfate	100	
Hydroxylamine hydrochloride	80	
Phenylmethanesulfonyl fluoride	0	0
Diisopropylfluorophosphate	9	5

a) A mixture of the enzyme solution (5.4 μg/0.9 ml) in 0.1 M Tris-HCl (pH 9.0) and 10 mM inhibitor solution (0.1 ml) in the same buffer was kept at room temperature for 20 min. The mixture was incubated with 1% casein solution (1 ml) in 0.1 M Tris-HCl (pH 9.0) at 37 °C for 20 min. The absorbance at 275 nm was measured against the blank after the addition of 10% TCA (3 ml) followed by centrifugation.

b) A mixture of the enzyme solution (12.5 μg/250 μl) in 25 mM Tris-HCl (pH 9.0) and 2 mM aqueous inhibitor solution (250 μl) was kept at room temperature for 20 min. The mixture was incubated with 5 mM Z–Gly–Pro–Leu–Gly solution (250 μl) in 25 mM Tris-HCl (pH 9.0). After the addition of 0.1 N AcOH (250 μl) and then Moore's ninhydrin solution (500 μl), the whole was kept at 100 °C for 10 min. The absorbance at 570 nm was measured against the blank.

TABLE V. Inhibition of Carrageenan-Induced Edema in Rat Hind Paw by Intraperitoneal Injection of Alkinonase S

Drug	*i.p.* dose mg/kg	No. of rat	Body weight g ± S.E.[a]	Edema, % ± S.E.[a] 1 h	3 h	5 h	Inhibition of edema, % 1 h	3 h	5 h
Alkaline proteinase S	5.0	5	128 ± 1.4	13.3 ± 2.31	16.0 ± 1.61	24.9 ± 3.34	68.1	79.9	74.2
	3.0	5	136 ± 1.9	24.4 ± 1.25	31.6 ± 1.70	53.2 ± 3.97	41.7	60.4	44.7
	1.0	5	131 ± 1.0	30.6 ± 1.85	39.2 ± 2.09	64.8 ± 7.15	26.9	50.9	32.6
	0.5	5	126 ± 2.4	35.0 ± 4.19	45.3 ± 2.19	69.7 ± 3.35	16.2	43.3	27.5
	0.1	5	127 ± 2.4	42.8 ± 4.99	67.5 ± 4.97	76.5 ± 4.61	0.0	15.5	20.5
Control		5	131 ± 2.5	41.8 ± 5.25	79.8 ± 6.81	96.2 ± 8.09			

a) Standard error.

TLC. The component obtained from the lower band was found to be DNS–Arg from the *Rf* values before and after HCl-hydrolysis. The component from the upper band was identified as DNS–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe based on the amino acid composition determined by Stein–Moore amino acid analysis of the HCl-hydrolysate. Under the conditions employed here, bradykinin was completely hydrolyzed.

Discussion

The alkaline metalloendopeptidase, alkinonase A, has been purified from the culture

fluid of *Streptomyces violaceorectus* MC 675-A8 by affinity chromatography on Z–Gly–Leu–AH–Sepharose,[2] and the same adsorbent was found to be effective for purifying a serine proteinase of the same origin, alkaline proteinase S, in this work. The enzyme showed amidase activity on Z–Gly–Leu–NH_2 (Table II), and this seemed to be the basis of the affinity of the enzyme for the adsorbent.

Besides endopeptidase activity (casein hydrolysis), alkaline proteinase S showed carboxypeptidase-like activity on bradykinin and Z–Gly–Pro–Leu–Gly (Table II). Both activities were inhibited by the same set of chemicals, as shown in Table IV. The hydrolysis rate of Z–Gly–Pro–Leu–Gly decreased in the presence of Z–Gly–Leu–NH_2 (Table II), suggesting that amidase activity and carboxypeptidase-like activity were due to the same molecule. Esterification of Z–Gly–Pro–Leu–Gly and addition of a proline residue to the N-terminal glycine increased the hydrolysis rate of this substrate by factors of 11.1 and 4.2, respectively. From these results, alkaline proteinase S seemed to act on appropriate substrates as a carboxypeptidase.

In respect of substrate specificity, alkaline proteinase S showed some similarity to alkaline proteinases a and c from *Streptomyces griseus*,[8] though no carboxypeptidase-like activity had been reported for the *Str. griseus* enzymes. While alkaline proteinases a and c had been reported to be stable in the pH range of 4.0—6.5,[8] alkaline proteinase S was stable at pHs from 5.0 up to 12.0 (Fig. 4). Anti-inflammatory activity of alkaline proteinase a from *Streptomyces griseoviridis* was reported by Nakamura *et al.*,[9] and the enzyme had characteristics quite similar to those of alkaline proteinase S except that the optimum pH of caseinolysis by alkaline proteinase a was 10.5—11.0.

According to Bauer *et al.*,[10] *Str. griseus* Protease 3 (SGP 3, identical with alkaline proteinase a of Narahashi *et al.*[8]) depends mainly on secondary enzyme–substrate contacts with amino acid residues P_2—P_4, remote from the scissile bond, whereas α-chymotrypsin depends mainly on primary contacts with amino acid residue P_1. As shown in Table II, almost negligible hydrolysis of Boc–Pro–Leu–Gly–NH_2 was observed in comparison with Z–Gly–Pro–Leu–Gly, Z–Gly–Pro–Leu–Gly–OEt and Z–Gly–Pro–Leu–Gly–Pro. It would be interesting to compare the contributions of primary and secondary contacts to the rates of hydrolysis of various synthetic substrates by alkaline proteinase S.

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