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An Neutral Proteinase obtained from *Streptomyces griseolus*[1)]

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Kinonase AI, AIII and BI produced by *Str. kinoluteus* and retikinonase I and II produced by *Str. verticillatus* are neutral proteinases having antibradykinin activity.[3–5)] Specially, retikinonase I shows remarkablely strong anti-inflammatory activity for the carrageenin-induced edema of rat hind paw.[5)] Besides, *Str. griseus*,[6)] *Str. fradiae*,[7)] *Str. caespitosus*,[8)] *Str. naraensis*,[9)] *Str. albus*,[10)] *Str. phaeochromogenes*,[8)] and *Str. ambopfaciens*[8)] are known as proteinase-producing *Streptomyces* and neutral proteinases have been isolated from the cultured broths of *Str. griseus*,[6)] *Str. fradiae*,[7)] *Str. caespitosus*,[8)] and *Str. naraensis*.[9)]

A neutral proteinase is isolated from the cultured broth of *Str.* MA 429-A2, identified to be *Str. griseolus*, and its isolation, purification and enzymatic studies are described in this paper.

Str. griseolus was cultivated in a jar fermentor using a medium composed of soy bean meal, glucose, starch and inorganic salts at 27—28° for 67 hr to produce the proteinases. The proteolytic mixture was recovered from the cultured broth by precipitation by saturation of ammonium sulfate, dialysis against water and following lyophilization. From the crude enzyme mixture, two proteinases were separated by gel filtration on Sephadex G 75. The first eluted proteinase fractions showed weak proteolytic activity and the most of proteolytic activity was eluated in the later eluted fractions. Each fraction was determined its purity by electrophoresis on a cellulose acetate film using 0.1 M Tris–HCl buffer (pH 7.0) for 30 min at 20 mA and 150 V and Ponceu 3R reagent was used to detect proteins. Four protein spots were observed in the first eluted weak proteolytic fractions at 6 mm, 8 mm, 10 mm and 11 mm to the cathode by the electrophoresis, while two protein spots at 11 mm and 14 mm to the same direction were shown in the later eluted proteinase fractions. Then, the later eluted proteinase was purified on a carboxymethyl (CM)-cellulose column eluted with a linear gradient of aqueous sodium chloride. The above eluted proteinase fractions were further purified by gel filtration on a Sephadex G 75 column to give a neutral proteinase which shows one spot at 11 mm to the cathode by the electrophoresis. The highly purified proteinase was obtained as white amorphous powder by the above mentioned procedure.

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4) S. Nakamura, Y. Marumoto, H. Miyata, I. Tsukada, N. Tanaka, M. Ishizuka, and H. Umezswa, *Chem. Pharm. Bull.* (Tokyo), **17**, 2044 (1969).
5) S. Nakamura, M. Hamada, M. Ishizuka, and H. Umezawa, *Chem. Pharm. Bull.* (Tokyo), **18**, 2112 (1970).
6) *a)* A. Hiramatsu and T. Ouchi, *J. Biochem.*, **54**, 462 (1963); *b)* Y. Narahashi and M. Yanagita, *ibid.*, **62**, 633 (1967); *c)* Y. Narahashi, K. Shibuya, and M. Yanagita, *ibid.*, **64**, 427 (1968).
7) *a)* J.J. Noval and W.J. Nickerson, *J. Bacteriol.*, **77**, 251 (1959); *b)* W.J. Nickerson and J.J. Noval, *Biochim. Biophys. Acta*, **77**, 73 (1963); *c)* W.J. Nickerson and S.C. Durand, *ibid.*, **77**, 87 (1963); *d)* K. Morihara, T. Oka, and H. Tsuzuki, *ibid.*, **139**, 382 (1967).
8) *a)* Y. Yokote, K. Kawasaki, J. Nakajima, and Y. Noguchi, *J. Agr. Chem. Soc. Japan*, **43**, 125 (1969); *b)* Y. Yokote and Y. Noguchi, *ibid.*, **43**, 132 (1969).
9) A. Hiramatsu, *J. Biochem.*, **62**, 353, 364 (1967).
10) D.J. Hirschman, J.M. Zametkin, and R.E. Rogers, *Am. Dyestuff Reptr.*, **33**, 353 (1944).

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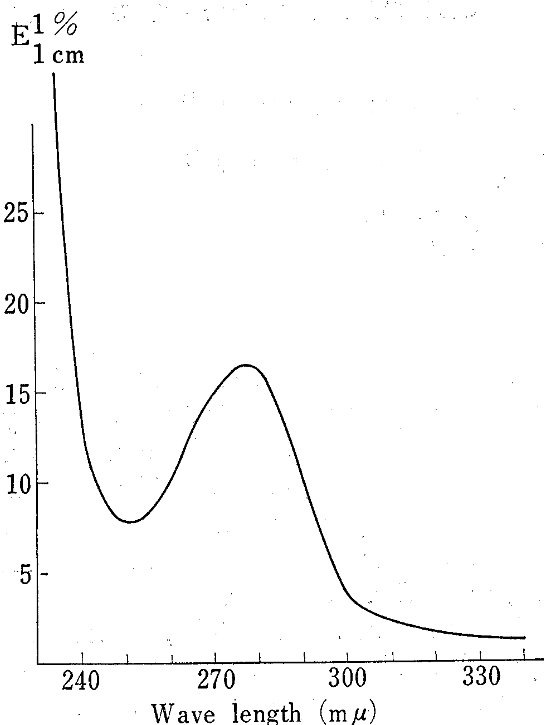

Fig. 1. Ultraviolet Spectrum in H_2O

The proteinase shows ultraviolet absorption maximum at 278 mμ ($E^{1\%}_{1cm}$=16.5) in water as shown in Fig. 1.

Effect of pH on proteolytic activity of the enzyme against casein is shown in Fig. 2 and the optimum pH is about 7.5—8.0. The proteinase is most stable at pH 7.0 and 80% of the activity is still reserved in a solution of pH 3.0 for 1 hr at room temperature as observed in Fig. 3. The maximum proteolytic activity is at 55° when incubated with casein at pH 7.0 for 20 min as shown in Fig. 4. The proteinase is table in 0.1 M Tris–HCl buffer (pH 7.0) at 50° for 10 min and 60% of the activity is lost at 60° for 10 min as shown in Fig. 5. Essentially no protective effect for the proteolytic activity is observed at the same condition when added 10^{-3} mole of calcium acetate. Effect of various enzyme inhibitors on the proteolytic activity of the enzyme is shown in Table I. Addition of calcium, ferrous, or lithium ion dose

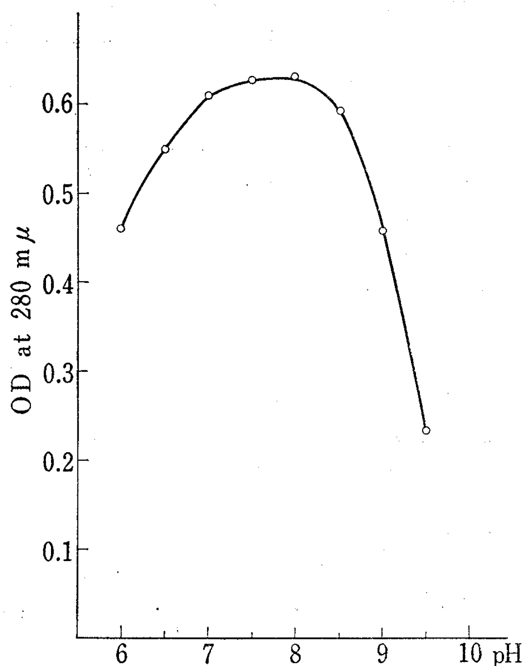

Fig. 2. Effect of pH on Proteolytic Activity

One ml of the aqueous enzyme solution (10 μg/ml) was incubated with 1 ml of 1% casein solution in 1/10 M Tris-HCl buffer (various pH's as indicated) for 20 min at 37°.

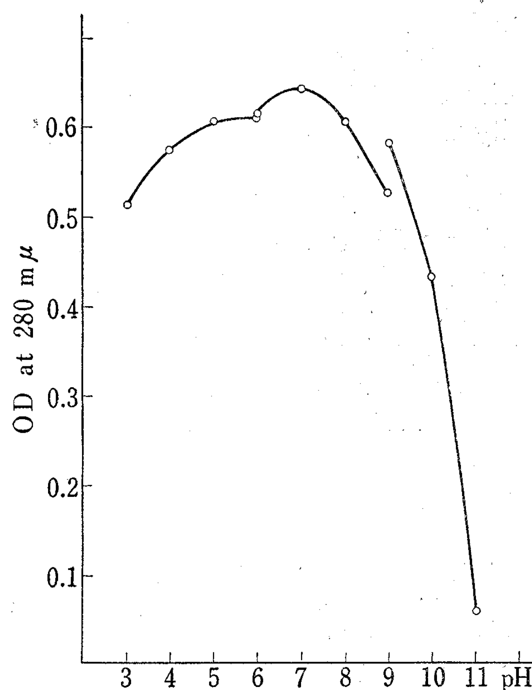

Fig. 3. pH Stability

1/10 M AcONa–AcOH buffer at pH 3—6, 1/10 M Tris–HCl buffer at pH 6—9 and 1/20 M borate buffer at pH 9—11 were used. A mixture of 0.1 ml of the aqueous enzyme solution (100 μg/ml) and 0.2 ml of the buffer was kept at room temperature for 1 hr. After adjusting the pH value to 7.0 and the total volume to 1 ml, the mixture was incubated with 1 ml of 1% casein solution in 1/10 M Tris–HCl buffer (pH 7.0) for 20 min at 37°.

not affect the proteolytic activity of the enzyme, but the activity is greatly reduced by addition of cupric or mercuric ion. Various enzyme inhibitors give similar effects on the proteolytic activity of the enzyme to that of kinonase AI, AIII, and BI, and retikinonase I and II. The

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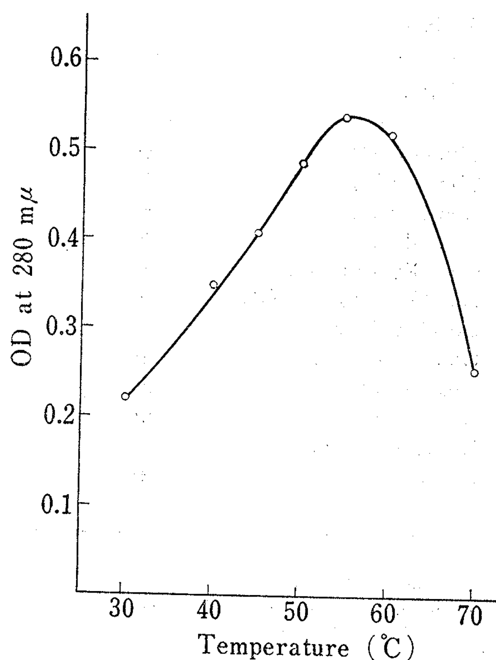

Fig. 4. Effect of Temperature on Proteolytic Activity

A mixture of 1 ml of the aqueous enzyme solution (6 μg/ml) and 1 ml of 1% casein solution in 1/10M Tris–HCl buffer (pH 7.0) was incubated at the test temperatures for 20 min.

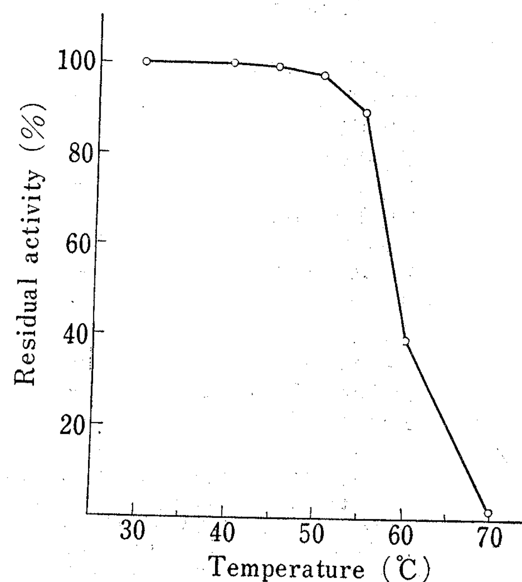

Fig. 5. Thermal Stability

One ml of the enzyme dissolved in 1/10M Tris–HCl buffer (pH 7.0) (10 μg/ml) was heated at test temperatures for 10 min. After cooling, the resulting solution was incubated with 1 ml of 1% casein solution in 1/10M Tirs–HCl buffer (pH 7.0) for 20 min at 37°.

Table I. Effect of Various Inhibitors on Proteolytic Activity

Metal ions	Residual activity (%)	Chemicals	Residual activity (%)
$AgNO_3$	77	ω-chloroacetophenone	100
$CaCl_2$	100	*p*-chloromercuribenzoate	69
$CoCl_2$	97	monoiodoacetic acid	81
$CuSO_4$	23	L-cystine	100
$FeSO_4$	100	ethylenediaminetetraacetate	4
$HgCl_2$	15	8-hydroxyquinoline	100
Li_2SO_4	100	diisopropylfluorophosphate	84
$MgCl_2$	96	sodium laurylsulfate	71
$MnCl_2$	78	iodine	2
$NaNO_2$	97	glutathione	100
$ZnSO_4$	89	glutathione-SSG	74
		N-bromosuccinimide	5
		cyanogen bromide	97
		hydroxylamine hydrochloride	75
		potato trypsin inhibitor[a]	100
		potassium permanganate	0

A mixture of 0.9 ml of the aqueous enzyme solution (11 μg/ml) and 0.1 ml of 1/100M inhibitor solution was kept for 20 min at room temperature. Then, the mixture was incubated with 1 ml of 1% casein solution in 1/10M Tris–HCl buffer (pH 7.0) for 20 min at 37°.
a) 0.1 ml of a solution (300 μg/ml) was used.

enzymatic activity of the proteinase is retained after addition of ω-chloroacetophenone, *p*-chloromercuribenzoate or potato trypsin inhibitor, but lost after addition of ethylenediamine tetraacetate, iodine or N-bromosuccinimide. The proteinase has similar substrate specificity to that of kinonase AI, AIII or BI or retikinonase I or II as shown in Table II and hydrolyzes the peptide bond in which the amino group of phenylalanine, tyrosine or leucine is contained.

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TABLE II. Substrate Specificities

Substrates		Substrates	
D,L-Ala-Gly-Gly	−	Cbz-Glu-Phe	+
D,L-Ala-Leu	−	Cbz-Glu-Tyr	±
Gly-Gly	−	Cbz-Gly-Leu	−
Gly-Leu	−	Cbz-Gly-Phe	±
Gly-Phe	−	Cbz-Gly-Phe-NH_2	+
Gly-Phe-NH_2	−	Cbz-Gly-Pro-Leu-Gly	±
Leu-Gly-Gly	−	Cbz-Phe-Tyr	−
Leu-β-naphthylamide	−	Cbz-Try-Leu-NH_2	+
Ac-Gly	−	Z-Glu-Tyr	±
Ac-D,L-Met	−	Z-Gly-Leu-NH_2	+
N-Ac-D,L-Try	−	Z-Gly-Pro-Leu	−
N-Ac-D,L-Tyr-OEt	−	Z-Pro-Leu-NH_2	+
Ac-D,L-Val	−		

All amino acids are L-form, unless otherwise specified.
A mixture of 1 ml of the aqueous enzyme solution (15 μg/ml) and 1 ml of 1/400M substrate solution in 1/100M Tris–HCl (pH 7.0) was incubated for 20 hr at 37° and lyophilized. The residue was dissolved in 0.1 ml of 50% methanol and chromatographed on silica gel H using BuOH–AcOH–H_2O=4:2:1. The digestion products were detected by spraying 1% ninhydrin solution.
+ : hydrolyzed, ± : weakly hydrolyzed, − : not hydrolyzed

The enzyme easily hydrolyzed the peptide bond of a N-substituted dipeptide having one of the above amino acids as the C-terminus in the form of an amide. When a N-substituted dipeptide contains one of the amino acids as the C-terminus having a free carboxyl group, the proteinase hydrolyzes the peptide bond to yield phenylalanine, weakly hydrolyzes to yield tyrosine and not hydrolyzes to yield leucine at the test condition. Thus, the proteinase has specificity to the amino acids in order of phenylalanine>tyrosine>leucine. The proteinase hydrolyzes bradykinin to arginylprolylprolylglycine, phenylalanylserylproline and phenylalanylarginine same as kinonase AI, AIII or BI or retikinonase I or II.

The anti-inflammatory activity of the proteinase will be reported in other paper with that of several neutral proteinases obtained from the cultured broths of *Streptomyces*.

Experimental

Assay of the Proteolytic Activity——The casein-280 mμ method was modified to determine the proteolytic activity as described in the previous paper.[3]

Production of the Proteinase——The inoculation seed was shake-cultured in a 500 ml shaking flask containing 80 ml of a medium composed of 1% soluble starch, 1% peptone, 1% molasses and 1% meat extract (pH 7.0) at 28° for 30 hr. The medium composed of 1% Prorich (soy bean meal distributed by Ajinomoto Co.), 1% glucose, 1% soluble starch, 0.1% K_2HPO_4, 0.05% $CaCl_2$ and 0.02% $ZnSO_4 \cdot 7H_2O$ (pH 7.4) was used for production of the proteinase in a stainless steel jar fermentor (30 liters). The inoculation seed (400 ml) above obtained by shaking culture was used to inoculate 20 liters of the production medium sterilized in the jar fermentor and cultivated for 67 hr at 27—28° under aeration (20 liters/min) and stirring (300 rpm). Defoam CA-220 (polyalkylene glycol produced by Nippon oil Co.) (30 ml/20 liters) was used for the production of the enzyme as an antifoaming agent. Proteolytic activity of the cultured broth at various periods was determined by the casein-280 mμ method and the maximum production was observed at 65 hrs cultivation.

Isolation of the Crude Proteinase——The cultured broth above obtained was filtered to remove the mycelium cake and the filtrate (18 liters) was saturated with $(NH_4)_2SO_4$ (8.5 kg) at 0° adjusting to pH 7.0 by addition of 1N NH_4OH. The mixture was kept in cool for 1 hr to complete precipitation of the proteinase and the proteinase thus precipitated was collected by cold centrifugation. The collected proteinase was then dialyzed in a cellophane tube against distilled water for 16 hr in cool and 4.83 g of the crude enzyme was recovered from the retantate by lyophilization recovering 25% of the proteolytic activity from the cultured broth.

Purification of the Proteinase——The crude proteinase (1.19 g) dissolved in H_2O (8 ml) was fractionated to each 15.5 ml of fraction on a column of Sephadex G 75 (48 cm × 2.2 cm diameter) eluted with water. Weak proteolytic activity was eluted in fractions 4—6 and most of the proteolytic activity was found in

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fractions 8—10. Lyophilization of fractions 4—6 gave 38 mg of a protein mixture, while 63 mg of a protein mixture was recovered from fraction 8—10. The electrophoresis on Separax (cellulose acetate film, 9 cm wide) using 0.1M Tris-HCl buffer (pH 7.0) was used for 30 min at 20 mA and 150 V to determine purity of the enzyme. Four purple red spots at 6, 8, 10 and 11 mm to the cathode due to proteins were detected on the protein mixture obtained from fractions 4—6 by treating with Ponceu 3R after the electrophoresis. The two spots, a main spot at 11 mm and a minor spot at 14 mm to the same direction, were appeared in the sample from fractions 8—10 by the same procedures. The protein mixture obtained from fractions 8—10 (60 mg) was dissolved in H_2O (20 ml) and applied to a column of CM cellulose (13 cm × 2 cm diameter) treated with 0.1M Tris-HCl buffer (pH 7.0) to separate two components. The column was eluted with a linear gradient of aqueous NaCl from 0 to 0.5M (total 560 ml) and the eluate was collected in 12.0 ml fraction. The proteolytic activity was eluted mainly in fractions 13—22 and the fraction 14 showed the maximum activity. Each fraction was dialyzed in a cellophane tube against distilled water for 3 hr and then lyophilized. The residues obtained from fractions 13—17 move 11 mm to the cathode as a single spot by the electrophoresis, while the residues from fractions 18—22 showed two spots at 11 mm and 14 mm to the same direction. The residues obtained from fractions 13—17 were dissolved in H_2O (3 ml) and further purified by gel filtration on a column of Sephadex G 75 (70 cm × 1.5 cm diameter) eluted with water. The eluate was collected in 11.3 ml and the pure proteinase showing one spot by the electrophoresis was recovered from fractions 11 (2 mg), 12 (5 mg) and 13 (3 mg) by following lyophilization.

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Neutral Proteinases produced by *Streptomyces cacaoi* var. *asoensis*[1)]

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Three neutral proteinases produced by *Str. kinoluteus*,[3,4)] two neutral proteinases produced by *Str. verticillatus* var. *zymogenes*[5)] and a neutral proteinase produced by *Str. griseolus*[6)] have been isolated, specially retikinonase I isolated from the cultured broth of *Str. verticillatus* var. *zymogenes* shows remarkablely strong anti-inflammatory activity for the carrageenin-induced edema of rat hind paw as reported in the previous paper.

Two neutral proteinases, fraction A and B, have been isolated from the cultured broth of *Str. cacaoi* var. *asoensis* which is known as the polyoxin producing strain.[7)] *Str. griseus*,[8)]

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5) S. Nakamura, M. Hamada, M. Ishizuka and H. Umezawa, *Chem. Pharm. Bull.* (Tokyo), presented.
6) S. Nakamura, M. Hamada and H. Umezawa, *Chem. Pharm. Bull.* (Tokyo), presented.
7) *a*) S. Suzuki, K. Isono, J. Nagatsu, T. Mizutani, Y. Kawashima and T. Mizuno, *J. Antibiotics* (Tokyo), **18**, 131 (1965); *b*) K. Isono, J. Nagatsu, Y. Kawashima and S. Suzuki, *Agr. Biol. Chem.* (Tokyo), **29**, 848 (1965); *c*) K. Isono and S. Suzuki, *ibid.*, **30**, 813, 815 (1966); *d*) S. Suzuki, K. Isono, J. Nagatsu, Y. Kawashima, K. Yamagata, K. Sasaki and K. Hashimoto, *ibid.*, **30**, 817 (1966).
8) *a*) A. Hiramatsu and T. Ouchi, *J. Biochem.*, **54**, 462 (1963); *b*) Y. Narahashi and M. Yanagita, *ibid.*, **62**, 633 (1967); *c*) Y. Narahashi, K. Shibuya and M. Yanagita, *ibid.*, **64**, 427 (1968).

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