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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1) This is Part V of "Anti-inflammatory Proteinases obtained from Streptomyces" by S. Nakamura.
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3) S. Nakamura, Y. Marumoto, H. Yamaki, T. Nishimura, N. Tanaka, M. Hamada, M. Ishizuka and H. Umezawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 714 (1969).
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Str. fradiae,[9] *Str. caespitosus*,[10] *Str. naraensis*,[11] are known as proteinase producing *Streptomyces*. Strong proteolytic activity has been determined in the cultured broths of *Str. albus*,[12] *Str. phaeochromogenes*[10] and *Str. ambofaciens*.[10] The proteinases, Fr. A and Fr. B, produced by *Str. cacaoi* var. *asoensis* show similar enzymatic characteristics to other neutral proteinases obtained from *Str. kinoluteus*, *Str. verticillatus* var. *zymogenes* and *Str. griseolus* but the proteinases Fr. A and Fr. B can be differenciated from those neutral proteinases of *Streptomyces* origin by the electrophoresis. Both proteinases hydrolyze bradykinin to arginylprolylprolylglycine, phenylalanylserylproline and phenylalanylarginine as same as kinonases or retikinonases.

Str. cacaoi var. *aseoensis* was cultivated in a fermentation tank using a medium containing starch, peptone, meat extract and various inorganic salts to produce the proteinases. The proteinases were precipitated from the cultured broth by saturation with ammonium sulfate and a crude enzyme mixture was precipitated by addition of acetone after dialysis against water. Two proteinases were separated from the crude enzyme mixture, Fr. A from earlier fractions and Fr. B from a little later fractions, by gel filtration on a column of Sephadex G 75 after purified by gradient column chromatography on DEAE-cellulose. The proteinase Fr. B was further purified by gradient column chromatography on CM-cellulose and by gel filtration on Sephadex G 75. The proteinase Fr. A moves 8 mm toward the cathode and Fr. B 12 mm to the same dirction after electrophoresis at 10 mA and 110 V on Separax (cellulose acetate film, 7.5 cm wide, 6 cm long) for 1 hr using 0.1 M tromethamine (Tris)-HCl buffer solution (pH 7.0). Kinonase AI and AIII move 14 mm toward the cathode, retikinonase I 10 mm toward the same direction, the neutral proteinase obtained from *Str. griseolus* 11 mm toward the same direction and kinonase BI 2 mm toward the anode by the electrophoresis.

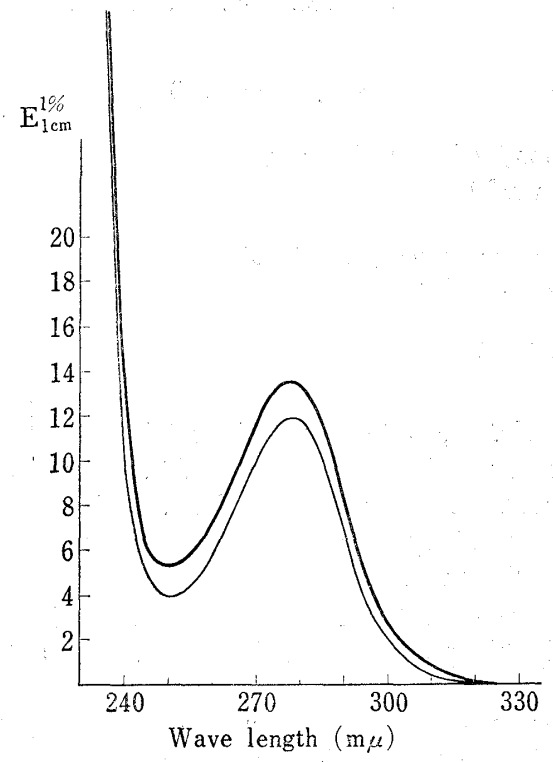

Fig. 1. Ultraviolet Spectra in H_2O

———: proteinase Fr. A
▬▬▬: proteinase Fr. B

The proteinases Fr. A and Fr. B show ultraviolet absorption maxima at 278 mμ in water as shown in Fig. 1. The effect of pH on the proteolytic activity of the proteinases Fr. A and Fr. B against casein is shown in Fig. 2. The optimum pH of the proteinase Fr. A is around 8 and that of Fr. B is around 6.5. Stability of both proteinases at various pHs is illustrated in Fig. 3. The proteinase Fr. A is rather stable at pH 6—9 at room temperature for 1 hr but more than 80% of the proteolytic activity of Fr. B is lost under the same condition at pH 9.0. The effect of temperature on the proteolytic activity of both proteinases is shown in Fig. 4. The optimum temperature for the proteinase Fr. A is around 50° when incubated with casein at pH 7.0 for 20 min, while that of Fr.

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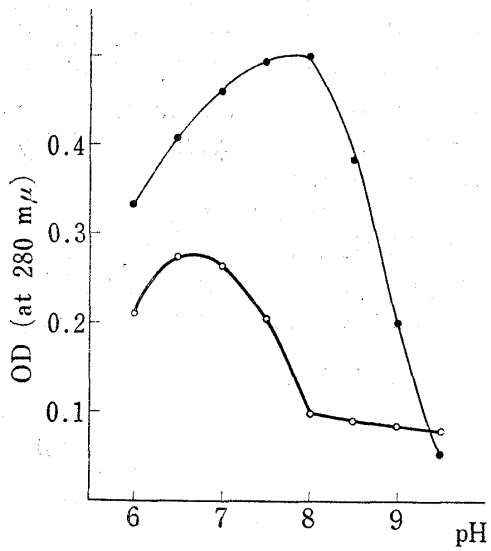

Fig. 2. Effect of pH on Proteolytic Activity

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°.

●——●: proteinase Fr. A
○——○: proteinase Fr. B

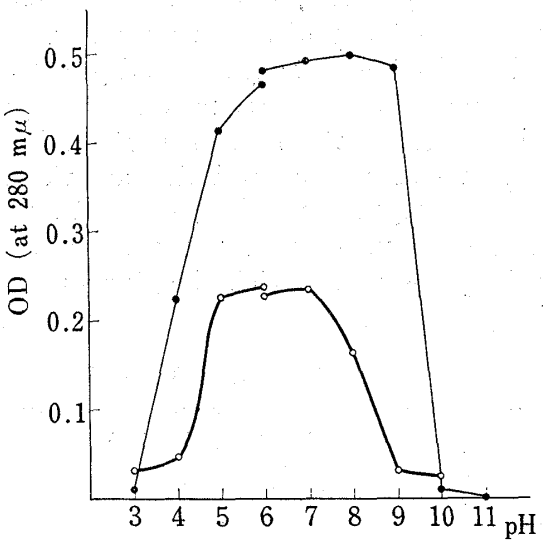

Fig. 3. pH Stability

1/10 M AcONa–AcOH buffer at pH 3—6, 1/10 M Tris-HCl buffer at pH 6—9 and 1/5 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°.

●——●: proteinase Fr. A
○——○: proteinase Fr. B

B is 35°. Thermal stability of the proteinases is shown in Fig. 5. The proteinase Fr. B is rather unstable and more than 20% of the activity is lost even at 30° for 10 min in 0.1 M Tris-

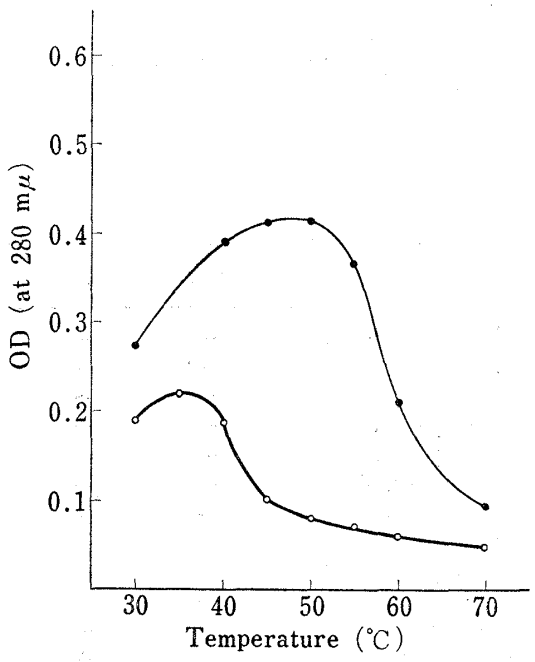

Fig. 4. Effect of Temperature on Proteolytic Activity

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

●——●: proteinase Fr. A
○——○: proteinase Fr. B

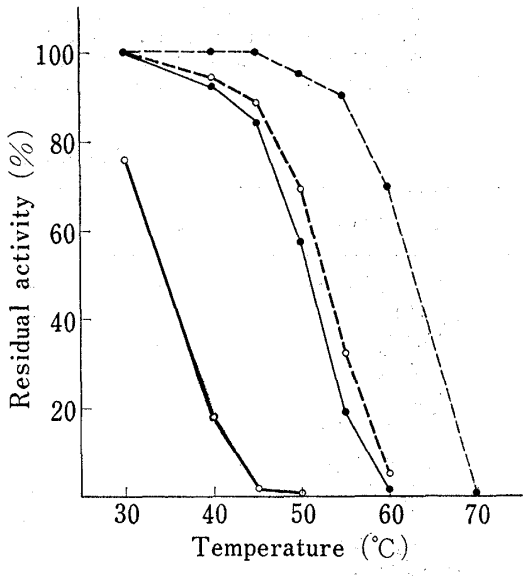

Fig. 5. Thermal Stability

One ml of the enzyme dissolved in 1/10 M 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 1/10 M Tris-HCl buffer (pH 7.0) for 20 min at 37°.

added 1/1000 M $(AcO)_2Ca$

●——● ●----------●: proteinase Fr. A
○——○ ○----------○: proteinase Fr. B

HCl buffer (pH 7.0), but addition of 0.001M $(AcO)_2Ca$ shows a protective action for the proteolytic activity of both proteinases. The effects of various metal ions and chemical inhibitors for the proteolytic activity of both enzymes are shown in Table I. Calcium or magnesium ion dose not reduce the proteolytic activity of the proteinases, while mercuric or cupric ion inactivates most of the activity of both proteinases. The enzymatic activity of both proteinases is retained after addition of ω-chloroacetophenone, *p*-chloromercuribenzoate and potato trypsin inhibitor, but lost after addition of ethylenediamine tetra-acetic acid. Substrate specificities of the proteinases are shown in Table II. Both enzymes have specificity toward phenylalanine, tyrosine and leucine and hydrolyze the peptide in which the amino gtoup of one of above acids is contained. The proteinase Fr. A or Fr. B hydrolyzes

Table I. Effect of Various Inhibitors on Proteolytic Activity

Metal ions	Residual activity (%)		Chemicals	Residual activity (%)	
	Fr. A	Fr. B		Fr. A	Fr. B
$AgNO_3$	46	17	ω-chloroacetophenone	98	90
$CaCl_2$	98	100	*p*-chloromercuribenzoate	100	100
$CoCl_2$	88	76	monoiodoacetic acid	94	70
$CuSO_4$	8	18	L-cysteine	100	91
$FeSO_4$	100	49	ethylenediaminetetraacetate	5	12
$HgCl_2$	0	0	8-hydroxyquinone	75	64
Li_2SO_4	100	86	diisopropylfluorophosphate	100	79
$MgCl_2$	100	94	sodium laurylsulfate	94	78
$NaNO_2$	100	82	iodine	5	6
$ZnSO_4$	70	62	glutathione (reduced)	94	88
			N-bromosuccinimide	22	15
			cyanogen bromide	100	100
			hydroxylamine hydrochloride	99	97
			potato trypsin inhibitor[a]	100	84
			potassium permanganate	0	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.

Table II. Substrate Specificities

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

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 1/400M 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

bradykinin to yield arginylprolylprolylglycine, phenylalanylserylproline and phenylalanylarginine. The mode of hydrolysis of bradykinin by the proteinases is same with that of kinonase AI, AIII or BI or retikinonase I or II or the neutral proteinase produced by *Str. griseolus*. The anti-inflammatory activity of the proteinases Fr. A and Fr. B will be reported in other paper with that of other neutral proteinases produced by *Streptomyces*.

Experimental

Assay for the Protelytic Activity——The casein-280 mμ method was modified to determine the proteolytic activity as des deseribed in the previous paper.[3]

Production of the Proteinases——An inoculation seed was shake-cultured for 24 hr at 27° in 500 ml shaking flask containing 80 ml of a medium composed of 1% glucose, 1% potato starch, 0.75% meat extract, 0.75% polypeptone, 0.5% $CaCO_3$, 0.3% NaCl, 0.1% $MgSO_4 \cdot 7H_2O$, 0.0008% $MnCl_2 \cdot 4H_2O$, 0.0007% $CuSO_4 \cdot 5H_2O$, 0.0002% $ZnSO_4 \cdot 7H_2O$ and 0.0001% $FeSO_4 \cdot 7H_2O$ (pH 7.0). A medium composed of 2% glucose, 2% potato starch, 1.5% meat extract, 1.5% polypeptone and the same amount of inorganic salts with the seed medium (pH 7.0) was used for production of the proteinases. The cultured seed (2 liters) was used to inoculate 130 liters of the production medium sterilized in a fermentation tank (200 liters) and cultured at 27° for 73 hrs under aeration (130 liters/min) and stirring (320 rpm). The proteolytic activity of the cultured broth at various periods was determined by the method used for kinonases.[3] The maximum production of the enzymes was obtained at 65 hr in the fermentation and hervested at 73 hr.

Isolation of the Crude Proteinases——The mycelial cake was removed by filtration and $(NH_4)_2SO_4$ (50 kg) was added to the broth filtrate (100 liters) at 0° adjusting to pH 7. The precipitated enzymes were collected by cold centrifugation and dialyzed for 5 hrs in a cellophane tube against running water. Cold acetone (11 liters) was added to the retentate (3.76 liters) and mixture was kept at 5° over night to precipitate the enzyme mixture. The precipitated enzymes were separated and dried to give a crude proteinase mixture (63.5 g) (yield 33.8%).

Purification of the Proteinases——The crude proteinase mixture (2.5 g) dissolved in 100 ml of H_2O was purified on a column of DEAE-cellulose (54 × 2.2 cm diameter) adjusted to pH 7.0 by treatment with 1N HCl and thenafter 0.1M Tris–HCl buffer (pH 7.0) at 5°. The enzymes were eluted with a linear gradient of aqueous NaCl from 0 to 0.5M (total 600 ml). Dark brown inpurities was absorbed on DEAE-cellulose and the eluate was collected in every 19.5 ml fraction. Fractions 9—28 were separately dialyzed in cellophane tubes for 2 hr against distilled water containing a trace of $(AcO)_2Ca$ at 0° and lyophilized. Fraction 9—12 (total 220 mg) was further separated into two neutral proteinases, Fr. A and Fr. B, by gel filtration on a column of Sephadex G 75 (90 × 1.5 cm diameter) eluted with water. The eluate was collected in 10.5 ml fractions and the proteinase Fr. A was recovered from fraction 10 (2.5 mg), 11 (3 mg) and 12 (1.3 mg) by following lyophilization. The proteinase Fr. B (5 mg) was collected from fraction 14 and 15. Another 18 mg of Fr. B was collected from the crude proteinase mixture by the same procedure and the combined proteinase Fr. B (23 mg) was further purified on a CM-cellulose column (33 × 2 cm diameter) treated with 0.1M Tris–HCl buffer (pH 7.0). The proteinase absorbed on CM-cellulose was eluted with a linear gradient of aqueous NaCl from 0 to 0.5M (total 600 ml). The eluate was fractionated to each 11.4 ml of fractions and the proteinase Fr. B containing NaCl (37 mg) was recovered from fractions 15—17 by the dialysis and lyophilization. Thus recovered Fr. B (37 mg) was purified by gel filtration on a column of Sephadex G 75 (90 × 1.5 cm diameter) and the highly purified proteinase Fr. B (8 mg) was recovered from the fraction 14 (11.4 mg).