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Alkaline Proteinases a and b produced by *Streptomyces griseoviridis*[1]

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Two alkaline proteinases, designated as alkaline proteinases a and b, are isolated from the cultured broth of *Streptomyces H 61B-SY 1*, calssified as *Streptomyces griseoviridis*. The optimum pHs of alkaline proteinases a and b for casein are 10.5—11 and 11—11.5 respectively. Production, isolation, purification, effect of pH on the proteolytic activity, pH stability, effect of temperature on the proteolytic activity, effect of enzyme inhibitors on the proteolytic activity, substrate specificities and anti-inflammatory activity for the carrageenin-induced edema of alkaline proteinases a and b are described.

Optimum pH of most alkaline proteinases of *Streptomyces* origin are 9—10, while proteinases A and B produced by *Streptomyces rectus*[3] and alkaline proteinase fraction Ib produced by *Streptomyces fradiae*[4] have been reported to show rather high optimum pH at 10.5—11.5. Alkaline proteinases, designated as alkaline proteinases a and b, are isolated from the cultured broth of *Streptomyces H 61* B-*SY 1*, one of *Streptomyces* screened for the alkaline proteinases.[1] Optimum pH of both isolated alkaline proteinases are 10.5—11.5. Production, isolation, purification, enzymatic studies and anti-inflammatory activity of alkaline proteinases a and b are reported in this paper.

The proteinase-producing strain, *Streptomyces H 61 B-SY 1*, was isolated from a soil sample collected at Nara prefecture. *Streptomyces H 61 B-SY 1* is classified to be *Streptomyces griseoviridis*.[5] Nevertheless, color of aerial mycelia of *Streptomyces H 61 B-SY 1* is more dark than that of *Streptomyces griseoviridis* and the former utilizes sucrose but the later dose not.

Streptomyces H 61 B-SY 1 was cultivated in a tank fermentor for 70 hr at 28—29° using a medium composed of glucose, potato starch, meat extract, peptone, soy bean meal and various metal salts to produce the alkaline proteinases. The modified casein-280 mμ method[1,6] was applied to determine the proteolytic activity for production, isolation, purification and enzymatic studies of the alkaline proteinases.

The active component was precipitated from the supernatant of cultured-broth by saturation with ammonium sulfate and the precipitate was dialyzed in a cellophane tube against distilled water. The crude enzyme mixture was precipitated from the dialyzed enzyme solution by addition of cold acetone. The crude powder containing alkaline proteinase activity was separated to two alkaline proteinases by gradient column chromatography on DEAE-

1) This is Part VII of "Anti-inflammatory Proteinases obtained from Streptomyces" by S. Nakamura. Part VI: S. Nakamura, H. Fukuda, T. Yamamoto, M. Ogura, M. Hamada, M. Matsuzaki, and H. Umezawa, *Chem. Pharm. Bull.*, (Tokyo), **20**, 385 (1972).
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3) *a*) K. Mizusawa, E. Ichishima, and F. Yoshida, *Agr. Biol. Chem.* (Tokyo), **28**, 884 (1964); *b*) K. Mizusawa E. Ichishima, and F. Yoshida, *Agr. Biol. Chem.* (Tokyo), **30**, 35 (1966).
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5) L.E. Anderson, J. Ehrlich, S.H. Sun, and P.R. Burkholder, *Antibiot. Chemotherapy*, **6**, 100 (1956); *b*) E.B. Shirling and D. Gottlieb, *International Journal of Systematic Bacteriology*, **18**, 332 (1968).
6) S. Nakamura, Y. Marumoto, H. Yamaki, T. Nishimura, N. Tanaka, M. Hamada, M. Ishizuka, T. Takeuchi, and H. Umezawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 714 (1969).

cellulose after gel filtration on a column of Sephadex G 75. The first eluated proteinase, a minor component, was designated as alkaline proteinase b and the later, a main component, was designated as alkaline proteinase a. Purity of the enzyme was examined by the electrophoresis on a cellulose acetate film using 0.1 M Tris-HCl buffer (pH 7.0) at 1 mA/cm for 1 hr and each enzyme was detected as a reddish spot by treatment with Ponceau 3R reagent. The fractions containing alkaline proteinase a showed two spots at 6 and 8 mm toward the cathode by the electrophoresis and other fractions containing alkaline proteinase b at 12 and 14 mm to the same direction. The later eluated enzyme was further purified by gel filtration on a column of Sephadex G 75 to give highly purified alkaline proteinase a showing one spot at 8 mm toward the cathode by the electrophoresis. Gradient column chromatography on CM-cellulose of the earlier eluated enzyme gave alkaline proteinase b showing one spot at 12 mm toward the cathode by the electrophoresis and further purified by gel filtration on a column of Sephadex G 75.

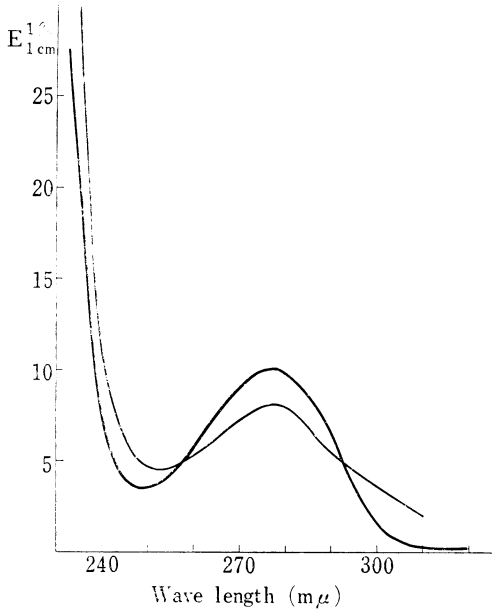

Fig. 1. Ultraviolet Absorption Spectra in H_2O

——: alkaline proteinase a
——: alkaline proteinase b

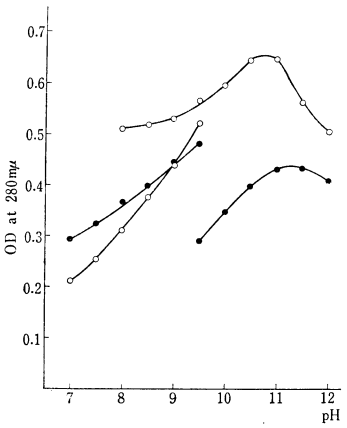

Fig. 2 Effect of pH on Proteolytic Activity

One ml of the aqueous enzyme solution (a=5 μg/ml, b=8 μg/ml) was incubated with 1 ml of 1% casein solution in M/10 Tris-HCl buffer (pH 6—9.5) or in M/20 borate buffer (pH 8—12) for 20 min at 37°.

—○—: alkaline proteinase a
—●—: alkaline proteinase b

The ultraviolet absorption spectra of alkaline proteinases a and b are shown in Fig. 1. The effect of pH on the proteolytic activity of both proteinases are shown in Fig. 2. The optimum pH of alkaline proteinase a is 10.5—11 and that of alkaline proteinase b is 11—11.5. Fig. 3 shows stability of both enzymes at various pH. Alkaline proteinase b is remarkably stable at pH 3—12 at room temperature for 1 hr and alkaline proteinase a is stable at pH 5—11 under the same condition. The effect of temperature on the proteolytic activity of both proteinases is shown in Fig. 4. The optimum temperature for alkaline proteinase a is 55—60° when incubated with casein at pH 9.0 for 20 min and that of the other is around 60°. Thermal stability of both proteinases heated 10 min at the tested temperature in M/200 borate buffer (pH 9.0) is shown in Fig. 5. The protective action by addition of 0.001 M $(AcO)_2Ca$ is observed specially for alkaline proteinase a. Table I shows effects of various metal ions and chemicals (10^{-3} M) on the proteolytic activity of alkaline proteinases a and b. The pro-

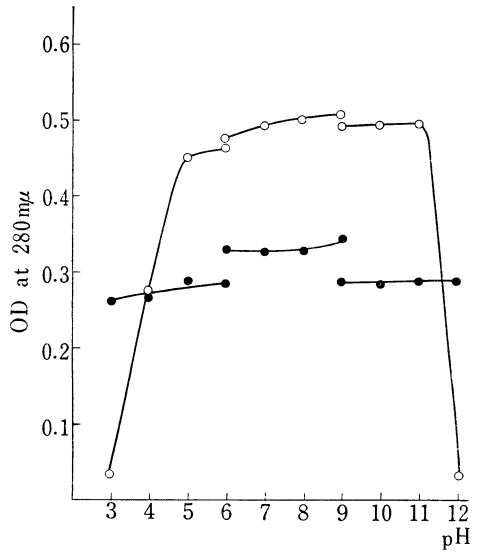

Fig. 3. pH Stability

M/10 Citrate buffer at pH 3—6, M/10 Tris-HCl buffer at pH 6—9 and M/20 borate buffer at pH 9—12 were used. A mixture of 0.1 ml of the aqueous enzyme solution (a=50 μg/ml, b=80 μg/ml) and 0.2 ml of the buffer was kept at room temperature for 1 hr. After adjusting the pH value to 9.0 and the total volume to 1 ml, the mixture was incubated with 1 ml of 1% casein solution in M/20 borate buffer (pH 9.0) for 20 min at 37°.

—○—: alkaline proteinase a
—●—: alkaline proteinase b

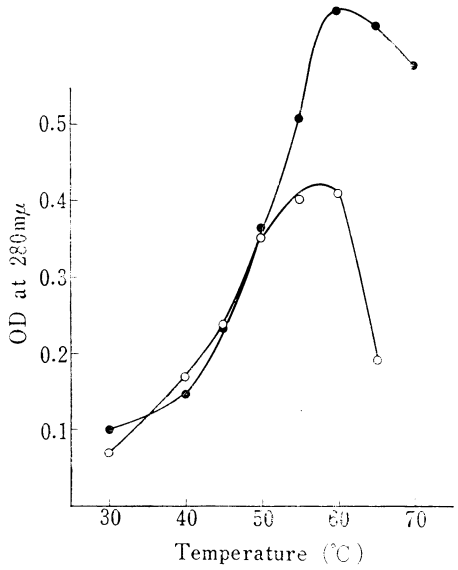

Fig. 4. Effect of Temperature on Proteolytic Activity

A mixture of 1 ml of the aqueous enzyme solution (a=2.5 μg/ml, b=4.0 μg/ml) and 1% casein solution in M/20 borate buffer (pH 9.0) was incubated at the test temperatures for 20 min.

—○—: alkaline proteinase a
—●—: alkaline proteinase b

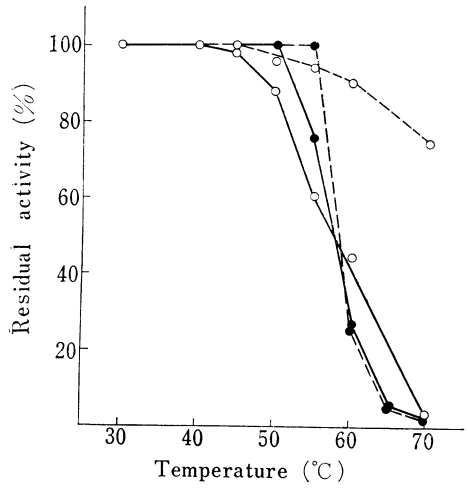

Fig. 5. Thermal Stability

One ml of the enzyme dissolved in M/200 borate buffer (pH 9.0) (a=5 μg/ml, b=8 μg/ml) was heated at the test temperatures for 10 min. After cooling, the resulting solution was incubated with 1 ml of 1% casein solution in M/20 borate buffer (pH 9.0) for 20 min at 37°.

—○—, ---○---: alkaline proteinase a
—●—, ---●---: alkaline proteinase b
added M/1000 (Ac C)$_2$Da

Table III. Inhibition of Carrageenin-Induced Edema in Rat hind Paw by the Alkaline Protease a

Dose	Inhibition of Edema (%)			
mcg/kg	1 hr	3 hr	5 hr	24 hr
200	25.0	29.6	29.2	21.9
50	18.8	19.4	16.0	12.3
12.5	21.9	17.3	16.0	11.0
3.1	15.6	4.1	5.7	5.5
0.625	21.9	5.1	8.5	8.2
Control Edema(ml)	0.32	0.98	1.06	0.73

route: *i.p.*
Carrageenin (1% solution 0.1 ml) was administered in rat hind paw after 1 hr of drug dosing.
rat: Male Wister Strain, 160—176 g.
Each figure is average of 5 rats on each dose level.

teolytic activity of both proteinases is retained after addition of *p*-chloromercuribenzoate, monoiodoacetic acid, ethylenediaminetetraacetate or soy bean trypsin inhibitor, but greatly inactivated after addition of mercuric chloride, diisopropylfluorophosphate or potato trypsin inhibitor. The substrate specificity of both proteinases are examined using various synthetic derivatives of amino acids and peptides as listed in Table II. None of the substrate is hydrolyzed by the proteinases except for Cbz-Gly-Pro-Leu-Gly is hydrolyzed by alkaline proteinase a. The anti-inflammatory activity of alkaline proteinase a was tested for the carrageenin-induced edema according to the method descrived in the previous paper.[1] The result is shown in Table III and alkaline proteinase a shows 30% inhibition of the edema by 0.2 mg/kg at 3 hr after injection of carrageenin.

The proteolytic activity of proteinase A and B produced by *Streptomyces* rectus[3] are almost completely inhibited after addition of *p*-chloromercuribenzoate at a concentration of

Table I. Effect of Various Inhibitors on Proteolytic Activity

Inhibitors	Residual activity(%) a	b	Inhibitors	Residual activity(%) a	b
$AgNO_3$	72	27	L-Cystine	100	85
$CaCl_2$	100	100	Ethylenediaminetetraacetate	100	90
$CoCl_2$	100	98	8-Hydroxyquinoline	100	100
$CuSO_4$	100	72	Diisopropylfluorophosphate	0	0
$FeSO_4$	52	89	Sodium laurylsulfate	100	78
$HgCl_2$	14	27	Iodine	0	0
Li_2SO_4	100	76	Glutathione	100	75
$MgCl_2$	100	100	N-Bromosuccinimide	0	0
$MnCl_2$	100	80	Cyanogen bromide	77	100
$NaNO_2$	100	100	Hydroxylamine hydrochloride	100	85
$ZnSO_4$	84	82	Potato trypsin inhibitor[a]	22	23
ω-Chloroacetophenone	73	78	Soy bean trypsin inhibitor[a]	100	78
p-Chloromercuribenzoate	100	97	Potassium permanganate	0	8
Monoiodoacetic acid	100	100			

A mixture of 0.9 ml of the enzyme dissolved in M/200 borate buffer (a=5 μg/ml, b=8 μg/ml) (pH 9.0) and 0.1 ml of M/100 inhibitor solution was kept for 20 min a room temperature. Then, the mixture was incubated with 1 ml of 1% casein solution in M/20 borate buffer (pH 9.0) for 20 min at 37°.
a) 0.1 ml of a solution (300 μg/ml) was used.

Table II. Substrate Specificities

Substrates	a	b	Substrates	a	b
D,L-Ala-Gly-Gly	—	—	N-Ac-Tyr-OEt	—	—
DL-Ala-Leu	—	—	Ac-D,L-Val	—	—
Gly-Gly	—	—	Cbz-Glu-Phe	—	—
Gly-Leu	—	—	Cbz-Glu-Tyr	—	—
Gly-Phe	—	—	Cbz-Gly-Leu	—	—
Gly-Phe-NH_2	—	—	Cbz-Gly-Phe	—	—
Leu-Gly-Gly	—	—	Cbz-Gly-Phe-NH_2	—	—
Ac-Gly	—	—	Cbz-Gly-Pro-Leu		—
Ac-D,L-Met	—	—	Cbz-Gly-Pro-Leu-Gly	+	—
N-Ac-Try	—	—	Cbz-Phe-Tyr	—	—
N-Ac-Try-OEt	—	—	Cbz-Try-Leu-NH_2	—	—

All amino acids are L-form, unless otherwise specified.
A mixture of 1 ml of the aqueous enzyme solution (a=5 μg/ml, b=8 μg/ml) and 1 ml of M/400 substrate solution in M/100 Tris-HCl buffer (pH 9.0) 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 G using BuOH:AcOH:H_2O=4:2:1. The digested substrate was detected by spraying 1% ninhydrin solution.
+: hydrolyzed, —: not hydrolyzed

10^{-3} M. Ferrous ion activates the proteolytic activity of both enzymes produced by *Streptomyces rectus*. The optimum pH of alkaline proteinase fraction Ib produced by *Streptomyces fradiae*[4] is 11—11.5 and its proteolytic activity is inhibited after addition of diisopropylfluorophosphate or potato trypsin inhibitor but not after addition of ethylenediaminetetraacetate or soy bean trypsin inhibitor. Nevertheless, stable pH of alkaline proteinase fraction Ib is 5—13 and that of alkaline proteinases a and b are respectively 5—11 and 3—12. Thus, alkaline proteinases a and b produced by *Streptomyces H 61 B-SY 1* can be differentiated from that of *Streptomyces* origin.

Experimental

Assay of the Proteolytic Activity——The modified casein-280 mμ method was used to determine the proteolytic activity as appeared in the previous paper[1,6] using 1% casein solution in 0.1 M Tris-HCl beffer (pH 7.0 and pH 9.5 unless otherwise described).

Production of the Alkaline Proteinases——An inoculation seed was cultured on a reciprocal shaker (120 rpm, amplitude 6 cm) at 29° for 48 hr using 500 ml shaking flasks containing each 100 ml of a medium composed of 3% glucose, 1% glycerol, 0.75% meat extract, 0.75% peptone, 0.5% aspartic acid, 0.2% yeast extract, 0.3% NaCl, 0.1% K_2HPO_4 and 0.1% $MgSO_4 \cdot 7H_2O$ (pH 6.7). The inoculation seed (2 liters) was used to inoculate a production medium (130 liters) composed of 1% glucose, 1% potato starch, 0.75% meat extract, 0.75% peptone, 0.5% soy bean meal, 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% $FeSO_4 \cdot 5H_2O$ (pH 7.6) sterilized in a stainless tank fermentor (200 liters). The tank fermentation was continued for 70 hr at 28—29° under aeration (130 liters/min) and stirring (200 rpm). Amtiforn KM 75 (0.08%) was used as an antiforming agent.

Isolation of the Crude Alkaline Proteinases——The mycelium cake was removed by centrifugation and $(NH_4)_2SO_4$ (44 kg) was added to the supernatant of cultured broth (110 liters, pH 8.3) adjusting pH 7.7—7.8. The precipitated enzyme mixture was collected by cold centrifugation after standing over night at 4° and the precipitate dissolved in H_2O (3 liters) was dialyzed in a cellophane tube against H_2O for 16 hr at 4°. Cold acetone (10.8 liters) was added to the dialyzate (4 liters) to precipitate the enzyme mixture. The precipitated crude mixture (85 g) was recovered by centrifugation followed by drying *in vacuo*.

Purification of the Alkaline Proteinases a and b——The crude alkaline proteinase mixture (21.4 g) dissolved in H_2O (30 ml) was dialyzed in a cellophane tube against distilled H_2O for 3 hr at 0°. Insoluble inpurity was removed by centrifugation and lyophilized to give 2.9 g of the residue. The residue (2.9 g) dissolved in H_2O (25 ml) was fractionated to each 14.5 ml of fractions on a column of Sephadex G 75. The fractions 5—10 were conbined and passed through a column of DEAE-cellulose (Cl^--type, 21 cm × 1.8 cm diam.) treated with 0.1M Tris-HCl buffer (pH 7.0). The column was eluted to each 18.3 ml of fractions with a linear gradient concentration of aqueous NaCl at a concentration from 0 to 0.25M (total 600 ml). The proteolytic activity was existed in fractions 5—8 (containing alkaline proteinase b) and fractions 16—22 (containing alkaline proteinase a). The active fractions were dialyzed respectively in cellophane tubes for 3 hr at 0° aginst distilled H_2O containing a trace of $(AcO)_2Ca$ and thereafter lyophilized to recover 340 mg of alkaline proteinase b from fractions 5—8 and 337 mg of a from fractions 16—22. Alkaline proteinase a (272 mg) dissolved in H_2O (5 ml) was separated by gel filtration on a column of Sephadex G 75 (97 cm × 1.5 cm diam.) eluted with H_2O and the eluate was collected in each 9.9 ml of fractions. The purified alkaline proteinase a (52 mg) was recovered from fractions 9—13. Alkaline proteinase b (340 mg) obtained from fractions 5—8 by DEAE-cellulose column chromatography was dissolved in H_2O (30 ml) and applied to a column of CM-cellulose (NH_4^+-type, 15 cm × 1.5 cm diam.) treated with 0.1M Tris-HCl buffer (pH 7.0). The column was eluted to each 11.5 ml of fractions with a linear gradient concentration of aqueous NaCl from 0 to 0.25M (total 600 ml) and the proteolytic activity was mainly eluted in fractions 18—20 and very weak proteolytic activity was in fractions 23—25. The active fractions were dialyzed respectively in cellophane tube against distilled H_2O containing a trace of $(AcO)_2Ca$ for 12 hr. Alkaline proteinase b (18 mg) from fractions 18—20 and a neutral proteinase (10 mg) from fractions 23—25 were recovered by following lyophilization. The alkaline proteinase (18 mg) recovered from fractions 18—20 dissolved in H_2O (4 ml) was fractionated to each 9.8 ml of fractions on a column of Sephadex G 75 (79 cm × 1.5 cm diam.) and the active component was eluted in fractions 12—15. Fractions 12 (2.5 mg), 13 (8.1 mg), 14 (5.6 mg) and 15 (5.6 mg) were lyophilized. Alkaline proteinase b (21.8 mg), thus obtained, was rechromatographed on a column of Sephadex G 75 (96 cm × 1.5 cm diam.) to collect each 9.6 ml of fractions and purified alkaline proteinase b (3.1 mg) was recovered from fractions 10—13.