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An Anti-inflammatory Proteinase, Kinonase AI and AIII obtained from a *Streptomyces*

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New anti-inflammatory proteinases named as kinonase AI and AIII, have been isolated as the active components from a culture filtrate of *Streptomyces* which showed antibradykinin activity. The *Streptomyces* was designated *Streptomyces kinoluteus* n. sp. Kinonase AI and AIII are precipitated by saturation with ammonium sulfate and purified by DEAE-cellulose or by Sephadex G-75 chromatography. Kinonase AI is a basic glycoprotein and kinonase AIII is a basic protein, but the enzymatic characters of both enzymes are similar each other. Kinonase AI and AIII are so-called neutral proteinases having optimum pH around 7.5 and optimum temperature at 50°. Kinonase AI and AIII are stable at pH 7—8, but most of their proteolytic activity is destroyed in an acidic solution or by heating at 60° for 10 min. Their proteolytic activity is destroyed by a heavy metal ion, ethylenediaminetetraacetate, N-bromosuccinimide or potassium permanganate, but not by ω-chloroacetophenone, 8-hydroxyquinoline, diisopropylfluorophosphate or potato trypsin inhibitor. Bradykinin is hydrolyzed by kinonase AI or AIII to arginylprolylprolylglycine, phenylalanylserylproline and phenylalanylarginine. Kinonase AI and AIII suppress the carrageenin-induced edema.

Bradykinin is one of important mediators to cause inflamation and culture filtrates of 1300 strains of microbes, mainly *Streptomycetes*, were tested for their antibradykinin activity. In this test, the mixture of a culture filtrate and bradykinin was injected intracutaneously to a rabbit which was intravenously injected with Evance Blue and the diameter of blue zone was measured. Eleven *Streptomycetes* strains and 2 bacterial strains showed the remarkable antagonistic activity to bradykinin. All of these active components were shown to be proteinases by their precipitation with ammonium sulfate, heating-unstability at 80° for 5 min, no dialysis through a cellophane tube and the proteolytic activity.

Proteinases obtained from microorganisms have different characteristics from those of animal or plant origin. Especially neutral proteinases of microbes possess specificity toward the amino acid residue of which the amino group is concerned in the peptide bond.[2,3]

Four proteolytic enzymes named as kinonase AI, AII, AIII and BI have been isolated from the cultured broth of *Streptomyces* M 993-C2 designated as *Streptomyces kinoluteus* n. sp. Kinonase AI, AIII and BI are neutral proteinases having the strong anti-inflammatory activity, while kinonase AII shows weak anti-inflammatory activity.

These neutral proteinases are differentiated from known proteinases of *Streptomyces* origin, that is, pronase,[4] keratinase,[5] prozyme,[6] and proteinases of *Streptomyces albus*,[7]

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2) K. Morihara, *Biochem. Biophys. Res. Commun.*, **26**, 656 (1957).
3) K. Morihara, H. Tsuzuki, and T. Oka, *Arch. Biochem. Biophys.*, **123**, 572 (1968).
4) *a*) M. Nomoto and Y. Narahashi, *J. Biochem.*, **46**, 653, 839, 1481, 1645 (1959); *b*) M. Nomoto, Y. Narahashi, and M. Murakami, *J. Biochem.*, **48**, 453, 593, 906 (1960); *c*) Y. Narahashi and M. Yanagita, *J. Biochem.*, **62**, 633 (1967); *d*) A. Hiramatsu and T. Ouchi, *J. Biochem.*, **54**, 462 (1963); *e*) Y. Narahashi, M. Yanagita, and K. Shibuya, The 19th Symposium on Enzyme Chemistry, Kanazawa, Apr. 1968.

Streptomyces fradiae,[8] and *Streptomyces naraensis*.[9] Characters of *Streptomyces kinoluteus*, production, and properties of kinonase AI and AIII are presented in this paper.

Streptomyces kinoluteus was isolated from a soil sample collected at Okukuji Park, Fukushima prefecture. Long straight aerial mycelia develope from fine branched substrate mycelia. Neither whorls nor spirals are observed. The surface of the spore observed by an electron microscope is generally smooth but some spores with flat warts. The cultural characteristics of the strain on various media are shown in Table I. Utilization of carbohydrates on the Pridham–Gottlieb basal medium is as follows: glycerol, xylose, fractose, galactose, glucose, mannose, maltose, dextrin and starch are utilized, yielding abundant growth; sucrose is utilized, yielding relatively good growth; arabinose, rhamnose, lactose, raffinose, sulicin, inulin, dulcitol, inositol, mannitol, sorbitol are not utilized, yielding no growth though faint growth is observed with raffinose.

Table I. Cultural Characteristics of *Streptomyces kinoluteus*

	Growth	Aerial mycelium	Soluble pigment	
Glycerol nitrate agar, 27°	dull yellow yellow orange	cottony, yellowish white to light gray	yellow to dull yellow	
Glucose-asparagine agar, 27°	pale yellow to yellow	cottony, yellowish white to light gray	yellowish	
Calcium malate agar, 27°	yellow to dull yellow	thin, white	yellowish	solubilization of Ca-malate: positive
Peptone solution (containing 1.0% of $NaNO_3$), 27°	pale yellow	none	yellowish	nitrate reduction: slight or none
Starch agar, 27°	yellow to dull yellow	white to light gray	yellow	hydrolysis of starch: weak
Tyrosine agar, 27°	yellow	scant, yellowish white	yellow	tyrosinase reaction: negative
Potato plug, 27°	yellow to pale yellowish brown	yellowish white	brownish	
Nutrient agar, 27°	pale yellow to yellowish brown	none	none	
Nutrient agar, 37°	colorless to pale brown	none	none	
Loeffler's serum, 37°	yellow to dull yellow	none	none	liquefaction of serum: negative
Gelatin, 20°	pale yellow to yellow	white	yellowish	liquefaction of gelatin: negative
Skimmed milk, 37°	colorless to pale brown ring	none	none	coagulation and slow peptonization
Cellulose, 27°	scant, no decomposition			

As shown by the characteristics described in Table I, *Streptomyces* M 993–C2 belongs to a nonchromogenic type, the growth on various media is yellow to dull yellow, the cottony aerial mycelium is yellowish to light gray, the soluble pigment is yellow, and the proteolytic action and hydrolysis of starch are weak. Among known species of *Streptomyces*, the strain is most similar to *Streptomyces chrysomallus* Lindenbein.[10] However, several differences are

5) J.J. Noval and W.J. Nickerson, *J. Bacteriol.*, 77, 251 (1959); W.J. Nickerson and J.J. Noval, *Biochim. Biophys. Acta*, 77, 73 (1963); W.J. Nickerson and S.C. Durand, *Biochim. Biophys. Acta*, 77, 87 (1963).
6) Y. Yokote and Y. Noguchi, The Annual Meeting of Agricultural Chemical Society of Japan, Sapporo, July 1964.
7) D.J. Hirschman, J.M. Zametkin, and R.E. Rogers, *Am. Dyestuff Reptr.*, 33, 353 (1944).
8) K. Morihara, T. Oka, and H. Tsuzuki, *Biochim. Biophys. Acta*, **139**, 382 (1967).
9) A. Hiramatsu, *J. Biochem.*, **62**, 353, 364 (1967).
10) S.A. Waksman, "The Actinomycetes," Vol. 4, Williams and Wilkins Co., Baltimore, 1961, pp. 142—193.

found between characters of *Streptomyces* M 993–C2 and *Streptomyces chrysomallus* as shown in Table II. Thus, *Streptomyces* M 993–C2 can be assigned to a new species named *Streptomyces kinoluteus*.

Table II. Comparison of *Streptomyces kinoluteus* with *Streptomyces chrysomallus*

	M993–C2	*S. chrysomallus*
Surface of spore	smooth or warty	smooth
Aerial mycelium	cottony, yellowish white to light gray	powdery, white
Hydrolysis of starch	weak	strong
Liquefaction of gelatin	negative	strong
Milk	coagulation and slow peptonization	coagulation and strong peptonization
Utilization of carbohydrate Rhamnose	not utilized	utilized
Metabolites produced	kinonase	actinomycin C and cycloheximide

Streptomyces kinoluteus was cultivated in a jar fermentor using a medium composed of soy bean meal, glucose, starch and several inorganic salts. The maximum proteolytic activity is obtained at 40 hours cultivation. A crude mixture of kinonases is recovered from the broth filtrate by vacuum condensation and salting out with ammonium sulfate followed by dialysis and lyophilization. The mixture of crude kinonases is separated on DEAE–cellulose to a mixture of kinonase AI and AIII, kinonase AII and kinonase BI. Kinonase AI and AIII are eluted with water from DEAE–cellulose, while kinonase AII and BI are absorbed on DEAE–cellulose and eluted with a saline solution of a gradient increasing concentration. Purity of kinonases can be examined by the electrophoresis on Separax (cellulose acetate film, Joko Sangyo Co.) using pH 7.0 buffer at 8 mV for 1 hr and each enzyme is detected as a reddish spot by treatment with Ponceu 3R reagent. Kinonase AI and AIII move by 1.5 cm to the cathode and kinonase AII moves by 0.8 cm to the same direction but kinonase BI moves by 0.2 cm to the anode on the electrophoresis. The casein–280 mμ method[11] was modified to measure the proteolytic activity of kinonases. Kinonase AI and AIII can not be distinguished by the electrophoresis but they are separated by gel filtration on Sephadex G–75 or G–200. Kinonase AI appears in earlier fractions by gel filtration on Sephadex G–75, while kinonase AIII appears in later fractions.

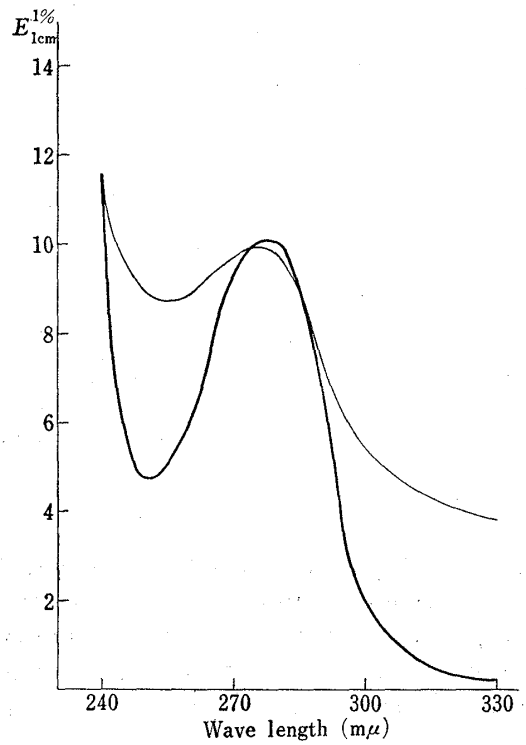

Fig. 1. UV Spectra of Kinonase AI and AIII

kinonase AI ——— kinonase AIII ———
kinonase AI; UV $\lambda_{max}^{H_2O(pH2)}$ mμ ($E^{1\%}_{1cm}$); 276—277 (9.9)
kinonase AIII; UV $\lambda_{max}^{H_2O}$ mμ ($E^{1\%}_{1cm}$); 278—280 (10.1)

11) B. Hagihara, N. Matsubara, J. Nakai, and K. Okunuki, *J. Biochem.*, **45**, 185 (1957).

Purified kinonase AI and AIII are obtained as white amorphous powder by repetition of the EDTA-cellulose treatment and the gel filtration.

Both of kinonases are of basic nature and kinonase AI is sparingly soluble in water, while kinonase AIII is easily soluble. The ultraviolet absorption spectra of kinonase AI and AIII are shown in Figure 1. Kinonase AI is suggested to be a glycoprotein by the infrared absorption spectrum and the low nitrogen content (N, 5.56%), while the infrared absorption spectrum of kinonase AIII does not show the bands at 1100—1000 cm^{-1} region due to sugar moieties. Hydrolysis of kinonase AI with constant boiling hydrochloric acid gives Asp, Thr, Ser. Glu, Pro, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe, Lys, His, NH_3 and Arg by the Stein-Moore amino acid analysis. The acid hydrolyzate of kinonase AIII also is shown to contain the same amino acids.

Proteolytic activity of kinonase AI and AIII at various pH against casein is shown in Figure 2. Optimum pH of kinonase AI and AIII are around 7.5. Thus, both kinonases can be classified into a category of so-called neutral proteinases. As shown in Figure 3, kinonase AI and AIII are most stable at pH 7—8 and lose their proteolytic activity in an alkaline or acidic solution. Especially it is unstable in an acid solution. The highest proteolytic activity is observed at 50° for both kinonases when incubated with casein at pH 7.0 for 20 min as indicated in Figure 4. Figure 5 shows thermal stability of kinonase AI and AIII. Table III and IV show effects of various metal ions and chemicals to proteolytic activity of kinonases. Proteolytic activity of kinonases is greatly decreased by heavy metal ions such as Cu^{2+} and Hg^{2+} as seen in Table III. As generally observed in neutral proteinases derived from microbes, proteolytic activity of both kinonases is not greatly inactivated by potato trypsin inhibitor, *p*-chloromercuribenzoic acid or diisopropylfluorophosphate, but inactivated

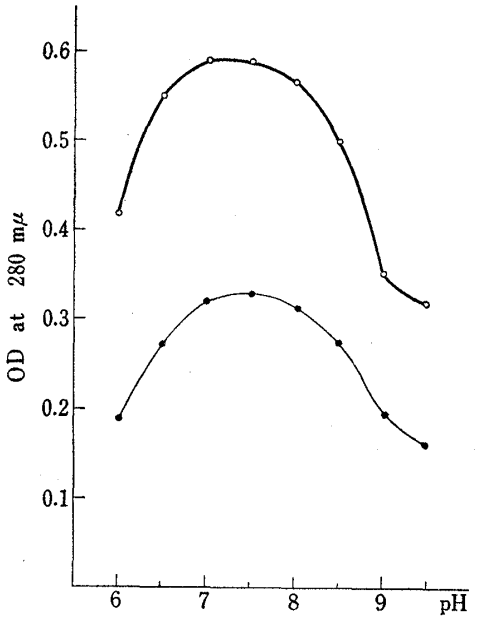

Fig. 2. Effect of pH on Proteolytic Activity of Kinonase AI and AIII

One ml of the aqueous enzyme solution (6 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°.
kinonase AI -●—●- kinonase AIII -○—○-

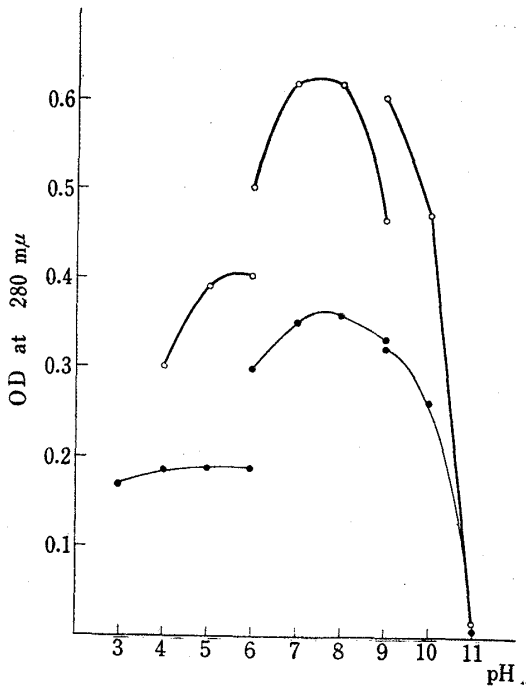

Fig. 3. pH Stability of Kinonase AI and AIII

1/10 M citrate buffer at pH 3—6, 1/10 M Tris-HCl buffer at pH 6—9 and 1/20M borate buffer at pH 9—11 were used. A mixture of 0.1 ml of the aqueous enzyme solution (60 μg/ml) and 0.2 ml of the buffer was kept for 1 hr at room temperature. After reversing 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°.
kinonase AI -●—●- kinonase AIII -○—○-

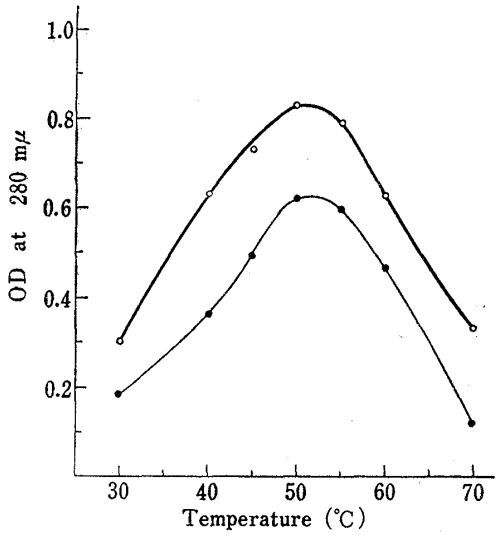

Fig. 4. Effect of Temperature on Proteolytic Activity of Kinonase AI and AIII

A mixture of 1ml 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 various temperatures for 20 min.
kinonase AI -●—●- kinonase AIII -○—○-

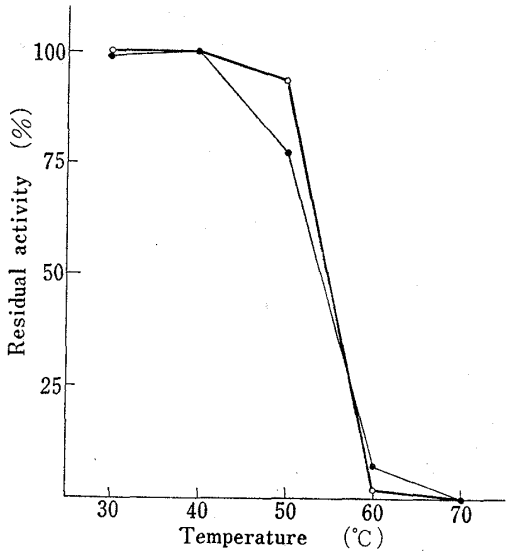

Fig. 5. Thermal Stability of Kinonase AI and AIII

One ml of the aqueous enzyme solution (6 μg/ml) was heated at various temperature for 10 min. After cooled, the resulting solution was incubated with 1 ml of 1% casein solution in 1/10M Tris–HCl buffer for 20 min at 37°.
kinonase AI —●—●— kinonase AIII —○—○—

by ethylenediamine tetra-acetic acid. L-Cystine increased proteolytic activity of both kinonases.

The substrate specificity of kinonases was studied using various synthetic derivatives of amino acids and peptides as shown in Table V. Kinonase AI and AIII are considered to hydrolyze the peptide bond in which the amino group of phenylalanine, tyrosine or leucine is concerned. α-Chymotrypsin, carboxypeptidase N, carboxypeptidase B and proteinase b

Table III. Effect of Metal Ions on the Activity of Kinonase AI and AIII

Metal Ions	Residual activity (%) Kinonase AI	Kinonase AIII
$AgNO_3$	53	54
$CaCl_2$	97	94
$CoCl_2$	84	65
$CuSO_4$	25	21
$FeSO_4$	82	85
$HgCl_2$	7	4
Li_2SO_4	100	100
$MgCl_2$	103	104
$MnCl_2$	77	74
$NaNO_2$	90	90
$ZnSO_4$	91	82

Table IV. Effect of Various Chemicals on the Activity of Kinonase AI and AIII

Chemicals	Residual activity (%) Kinonase AI	Kinonase AIII
ω-Chloroacetophenone	98	100
p-Chloromercuribenzoate	42	62
Monoiodoacetic acid	28	37
L-Cystine	135	120
Ethylenediaminetetraacetate	7	2
8-Hydroxyquinoline	100	100
Diisopropylfluorophosphate	78	80
Sodium lauryl sulfate	90	83
Iodine	56	23
Glutathione	32	39
Glutathione-SSG	33	48
N-Bromosuccinimide	3	1
Cyanogen bromide	99	95
Hydroxylamine hydrochloride	87	57
Potato trypsin inhibitor[a]	100	100
Potassium permanganate	0	0

effects of various inhibitors on proteolytic activity of kinonase AI and AIII

A mixture of 0.9 ml of the aqueous enzyme solution (6.7 μg/ml) and 0.1 ml of 1/100 M inhibitor solution was kept for 20 min at room temperature. Then, 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°.
a) 0.1 ml of a solution (180 μg/ml) was used.

Table V. Substrate Specificities of Kinonase AI and AIII

Substrates	Kinonase AI	Kinonase AIII
D,L-Ala-Leu	−	−
D,L-Ala-Gly-Gly	−	−
Gly-Gly	−	−
Gly-Leu	−	−
Gly-Phe	−	−
His-Phe-Arg-Try-Gly	+	+
Leu-Gly-Gly	−	−
Leu-β-Naphthylamide	−	−
Leu-Phe-OMe	−	−
Ser-Tyr-Ser-Met	+	+
Ac-Gly	−	−
N-Ac-Try	−	−
N-Ac-D,L-Try-OEt	−	−
N-Ac-Tyr-O-Et	−	−
Ac-D,L-Val	−	−
Cbz-Gly-Leu	−	+
Cbz-Gly-Phe	+	+
Cbz-Gly-Phe-NH_2	+	+
Cbz-Gly-Pro-Leu-Gly	±	+
Cbz-Glu-Phe	±	+
Cbz-Glu-Tyr	+	+
Cbz-Phe-Tyr	−	±
Cbz-Try-Leu-NH_2	+	+

All amino acids have the L-form, otherwise specially described.

A mixture of 1 ml of the aqueous enzyme solution (10 μg /ml) and 1 ml of $^1/_{400}$ M substrate solution in $^1/_{100}$ M 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 was applied to silicagel H thin-layer chromatography using BuOH–AcOH–H_2O=4:2:1. The digested substrate was detected by splying 1% ninhydrin solution.

+: hydrolyzed −: not hydrolyzed

are known to cleave bradykinin. Their modes of action on bradykinin are illustrated in Table VI. The mode of action of kinonase AI and AIII are different from that of the above enzymes, as seen in the same table. Bradykinin is hydrolyzed by kinonase AI or AIII to arginylprolylprolylglycine, phenylalanylserylproline and phenylalanylarginine. Dansyl derivatives of the three peptides are separated by the thin-layer chromatography of silica gel H and the constituent DNS–amino acid and amino acids involved in each DNS–peptide are identified by the thin-layer chromatography or the Stein–Moore amino acid analysis of the hydrolyzates.[12] Thus, kinonase AI and AIII are neutral proteinases and can be postulated to hydrolyze the peptide bonds of the amino groups of two phenylalanine.

Table VI. Modes of Action of Kinonase AI, AIII and Other Enzymes on Bradykinin

	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Kinonase AI	↑ ↑
Kinonase AIII	↑ ↑
α-Chymotrypsin	↑
Carboxypeptidase N	↑
Carboxypeptidase B	↑
Proteinase b	↑

12) Z. Tamura and T. Nakajima, *Protein, Nucleic Acid, Enzyme* (Tokyo), **12**, 21 (1967).

Anti-inflammatory effect of kinonase AI and AIII was tested for carrageenin-induced edema in hind paw of the rat.[13] For comparison, pronase P, bacterial alkaline proteinase and α-chymotrypsin were also examined for the anti-inflammatory effect. The results are shown in Table VII. Kinonase AI or AIII at 5.0 mg/kg showed the similar strength of the anti-inflammatory effect as bacterial proteinase (5 mg/kg) and a stronger effect than pronase P (5 mg/kg) and α-chymotrypsin (100 mg/kg).

Table VII. Anti-inflammatory Effect of Kinonase AI, AIII and Various Enzymes on Carrageenin-induced Edema

	mg/kg	Increased thickenss of edema (mm)	Inhibition (%)
Bacterial alkaline proteinase	5.0	1.4	72.7
Saline	—	5.1	0
α-Chymotrypsin	100	1.7	66.7
Kinonase AI	5.0	1.2	76.5
	1.25	2.9	43.2
Kinonase AIII	1.0	2.0	60.8
	0.25	2.8	45.1
Pronase P	5.0	2.7	47.5

at 4 hours after the injection of carrageenin

Thus, kinonase AI and AIII can be classified to the category of neutral proteinases from view points of their optimal pH, behavior to the enzyme inhibitors and substrate specificities. The enzymatic properties of kinonase AI and AIII resemble to that of microbial origin, nevertheless kinonase AI and AIII can be differentiated from known proteinases of *Streptomyces* origin.

The existence of seven proteinases, three aminopeptidases and one carboxypeptidase have been reported in pronase.[4e] The neutral proteinases of pronase, namely R-F-1-a purified on DEAE-Sephadex and F-2 isolated on CM-cellulose, are stable at pH 4—9 or by heating at 60° for 10 min. Keratinase is an alkaline proteinase.[5] The behavior of prozyme to various enzyme inhibitors is different from that of kinonase AI and AIII, especially 95% of the proteolytic activity of prozyme is destroyed by diisopropylfluorophosphate.[6] Only few description have been reported on the proteinase produced by *Streptomyces albus*.[7] *Streptomyces fradiae* produces the several proteinases. The fractions I-a, I-b and II are alkaline proteinases, while the fractions III and IV show the maximum proteolytic activity at pH 6.5—9 and more than thirty per cent of their proteolytic activity are retained by addition of 10^{-3} M of ethylenediaminetetraacetate.[8] The neutral proteinase of *Streptomyces naraensis* is of acidic nature and absorbed on DEAE-cellulose. This neutral proteinase shows the maximum proteolytic activity at 40°.[9] Thus, kinonase AI and AIII can be differentiated from known proteinases of *Streptomyces* origin.

Experimental

Assay of the Proteolytic Activity of Kinonases——The casein-280 mμ[11] method was modified to determine proteolytic activity of kinonases. A mixture of 1 ml of an enzyme solution and 1 ml of 1% casein solution in 0.1 M Tris-HCl buffer (pH 7.0 unless otherwise described) was incubated at 37° (unless otherwise described) for 20 min. The reaction mixture was cooled to 0° after the incubation and 3 ml of 5% trichloroacetic acid was added to the mixture to precipitate protein moieties. The precipitate was removed by centrifugation of 3000 rpm for 15 min and the optical density at 280 mμ of the supernatant was read by a spectrophotometer. A control test was also made by the same procedures using an inactivated enzyme

13) C.A. Winter, E.A. Risley, and G.W. Nuss, *Proc. Soc. Exptl. Biol. Med.*, **111**, 544 (1962).

solution heated at 80° for 10 min. The optical density at 280 mμ in the above method is parallel to a concentration of the enzyme solution lower than 12 μg/ml.

Production of Kinonases——A medium composed of 1% Prorich (soy bean meal of Ajinomoto Co.), 1% glucose, 1% starch, 0.3% NaCl, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.08% $MnCl_2 \cdot 4H_2O$, 0.02% $ZnSO_4 \cdot 7H_2O$, 0.01% $FeSO_4 \cdot 7H_2O$ and 0.007% $CuSO_4 \cdot 5H_2O$ (pH 7.0) was used for preparation of the inoculation seed and production of kinonases. The inoculation seed was cultured in a shaking flask of 500 ml volume containing 100 ml of the medium at 28° for 48 hr. The cultured broth of 400 ml was inoculated to 20 liters of the same medium sterilized in a stainless steel jar fermentor (30 liters) and cultivated at 27° under aeration of 20 liters/min and stirring of 300 rpm. Desform CA-220 (polyalkylene glycol of Nippon Oil Co.) was added at 27 ml/20 liters to the medium as an antiforming agent. Proteolytic activity at various periods of the cultured broth was determined and the maximum proteolytic activity was obtained at 40 hours fermentation.

Isolation of Crude Kinonases——The cultured broth (17 liters) was adjusted to pH 7.0 after removal of the mycelium cake by centrifugation and concentrated to 2.8 liters at 30—35° *in vacuo*. A mixture of kinonases was precipitated from the concentrate by addition of $(NH_4)_2SO_4$ (1.4 kg) at 0°. The precipitated mixture of kinonases was collected by cold centrifugation and then dialyzed in a cellophane tube against distilled water for 16 hours and 33 g of the crude mixture of kinonases was recovered by lyophilization.

Purification of Kinonase AI and AIII——The mixture of crude kinonases (15 g) was dissolved in H_2O and passed through a column of DEAE-cellulose (1.8 cm × 45 cm) and the column was eluted with NaCl solution of gradient concentrations from 0.05M to 0.5M. The eluates were fractionated to each 20 ml and the fractions were dialyzed in cellophane tubes for 4 hours against distilled water at 0°. Kinonase AI, AII and AIII were recovered from the fractions 5—15 by lyophilization and other kinonases from the fractions 16—22. The mixture of kinonases AI, AII and AIII was again applied to a column of DEAE-cellulose (1.8 cm × 7 cm) and eluted with distilled water. Kinonase AI and AIII were passed through the column and kinonase AII was absorbed on the column. Thus, kinonase AI and AIII (80 mg) were obtained from the eluate by lyophilization. The mixture of kinonase AI and AIII was dissolved in distilled water and separated to each component on a column of Sephadex G-75 (1.6 cm × 90 cm). The column was eluted with distilled water and the eluates were fractionated by each 8 ml. Kinonase AI was obtained from the fractions 6 (13 mg), 7 (19 mg) and 8 (9 mg), while kinonase AIII from the fractions 21 (4 mg), 22 (9 mg) and 23 (2 mg) by lyophilization.

Hydrolysis of Bradykinin by Kinonase AI and AIII——A mixture (pH 7.2) of 0.6 ml of a bradykinin solution (0.3 mg/ 0.6 ml) and 1 ml of a kinonase AI solution (0.01 mg/ml) was kept at 37° for 18 hours and evaporated to dryness *in vacuo*. The residue dissolved in 0.1 ml of 0.1M triethylamine bicarbonate was reacted with 0.4 ml of 1% dansylchloride solution in acetone for 18 hours at room temperature. The resulting DNS-peptides were separated to the three bands (*Rf*=0.63, 0.31, 0.17) on a thin-layer plate of silicagel H with iso-PrOH-AcOEt-28% NH_4OH=7:9:4. Each component eluated with acetone-H_2O-pyridine-AcOH=50:50:1:3 from silicagel H was hydrolyzed with constant boiling HCl in a sealed tube at 90° for 18 hours. The hydrolyzate from the upper band was shown to give DNS-Phe and each one mole of Ser and Pro, that of the middle band DNS-Phe and Arg, while the lowest DNS-Arg, one mole of Gly and two mole of Pro by the thin-layer chromatography of silica gel H with iso-PrOH-AcOEt-28% NH_4OH=7:9:4 or the Stein-Moore amino acid analysis. The same result was obtained by kinonase AIII.

Anti-inflammatory Effect of Kinonase AI, AIII and Various Proteinases on Carrageenin Induced Edema——To 3 rats (Donryu strain) weighing 80—100 g, 0.5 ml of a test proteinase was intraperitoneally injected and 1 hour later 0.05 ml of 1% carrageenin (Lot 520805, purchased from Marine Colloids Inc.) in saline was subcutaneously injected to their hind paws. Four hours thereafter, thickness of the edema was measured.

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