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An Anti-inflammatory Proteinase, Kinonase BI obtained from *Streptomyces kinoluteus*[1]

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A new anti-inflammatory proteinase designated as kinonase BI has been isolated from the mixture of kinonases produced by *Streptomyces kinoluteus*. Kinonase BI is purified by gradient column chromatography on DEAE–cellulose and gel filtration on a column of Sephadex G–75. Kinonase BI is considered to be a neutral proteinase of microbial origin. Kinonase BI is of acidic protein nature having pH optimum at 6.5 and optimum temperature at 55° against casein. Kinonase BI is rather stable in a solution of pH 5—9, but labile specially of acidic pH or by heating at 70° for 10 min. The proteolytic activity of kinonase BI is almost lost by addition of 10^{-3}M of a heavy metal ion, Cu^{2+} or Hg^{2+}, and ethylenediaminetetraacetic acid, but not by addition of ω-chloroacetophenone, *p*-chloromercuribenzoate diisopropylfluorophosphate and potato trypsin inhibitor at the same concentration. Kinonase BI hydrolyzes bradykinin to arginylprolylprolylglycine, phenyalanylserylproline and phenylalanylarginine. The anti-inflammatory activity on carrageenin-induced edema is observed in kinonase BI at the same level as in kinonase AI or AIII.

Kinonase AII containing a trace of kinonase AI is recovered also from the mixture of kinonases. Kinonase AII is presumed to be a kind of leucine amino peptidases. Bradykinin is not hydrolyzed by kinonase AII and the anti-inflammatory activity is not observed in kinonase AII.

Eleven Streptomyces and two bacterial strains among of 1300 microbes produced proteinases having the anti–bradykinin activity. As reported in the previous paper,[3] kinonase AI and AIII were isolated from the culture broth of *Streptomyces kinoluteus*. Both proteinases were proved to have a strong anti-inflammatory activity to carrageenin–induced edema in hind paw of rats. Kinonase AI is a glycoprotein, while kinonse AIII is a simple protein. Kinonase AI and AIII are so–called microbial neutral proteinases of basic protein nature and hydrolyze the peptide bond in which the amino group of phenylalanine, tyrosine or leucine is concerned.[3-5] The mode of hydrolysis of kinonase AI or AIII on bradykinin is different from those of α-Chymotrypsin, carboxypeptidase B, carboxypeptidase N, Kinonse II, and proteinase b which are known as bradykinin-hydrolyzing enzymes. Bradykinin is hydrolyzed to arginylprolylprolylglycine, phenylalanylserylproline and phenylalanylarginine by kinonase AI or AIII.

A new neutral proteinase of acidic protein nature, designated as kinonase BI, was also isolated from the crude kinonase mixture and its isolation and properties are presented in this paper.

1) This forms Part II of "Anti-inflammatory Proteinases obtained from Streptomyces."
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3) 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).
4) K. Morihara, *Biochem. Biophys. Res. Commun.*, **26**, 656 (1967).
5) K. Morihara, H. Tsuzuki and T. Oka, *Arch. Biochem. Biophys.*, 123, 572 (1968).

A crude kinonase mixture[3] is separated on a DEAE-cellulose column. Kinonase BI and AII are absorbed on DEAE-cellulose, while kinonase AI and AIII are passed through the column with water. Kinonase BI and AII absorbed on DEAE-cellulose are eluted with increase of the concentration of a saline. Purity of the proteinases is determined by the electrophoresis and the proteolytic acitivity is measured by the modified casein-280 mμ method.[3] Crude kinonase BI and AII thus eluted from the DEAE-cellulose column are further purified by gel filtration on Sephadex G-75. Purified Kinonase BI is recovered as white amorphous powder by repetition of gradient concentration column chromatography on DEAE-cellulose with aqueous sodium chloride and gel filtration on Sephadex G-75. Kinonase AII contaminated with a trace of kinonase AI is recovered from the earlier fractions than kinonase BI by gel filtration on Sephadex G-75. Kinonase BI has acidic protein nature and moves to the anode by 0.2 cm as a single spot in the electrophoresis on Separax at pH 7.0 8 mA for 1 hr, while kinonase AI or AIII moves 1.5 cm and kinonase AII 0.8 cm to the cathode. Kinonase BI: UV; H_2O mμ ($E^{1\%}_{1cm}$), 277—278 (19.4).

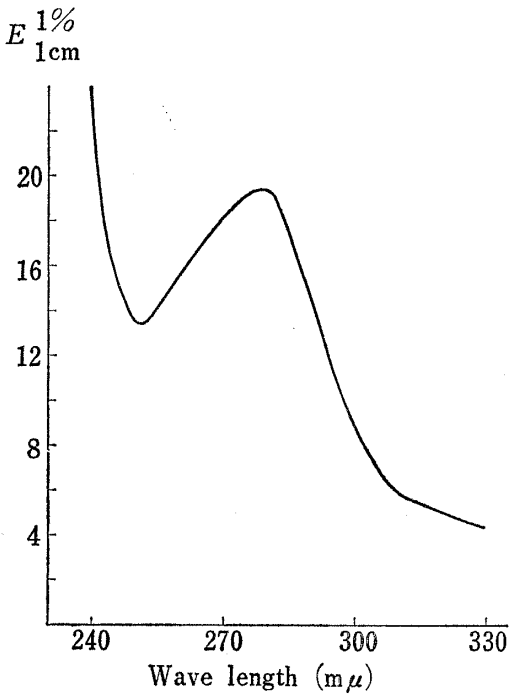

Fig. 1. UV Spectrum of Kinonase BI (in H_2O)

Kinonase BI has the same constituent amino acids with kinonase AI and AIII. Hydrolysis of kinonase BI with constant boiling hydrochloric acid yields 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

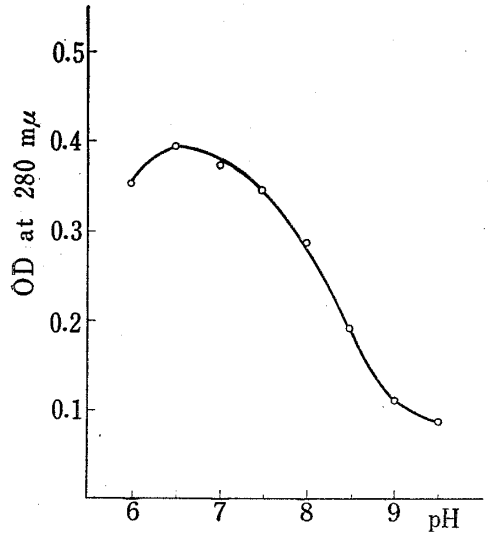

Fig. 2. Effect of pH on Proteolytic Activity of Kinonase BI

One ml of the aqueous enzyme solution (6 μg/ml) was incubated with 1 ml of 1% casein solution in 1/10M Tris-HCl buffer (various pH's as indicated) for 20 min at 37°

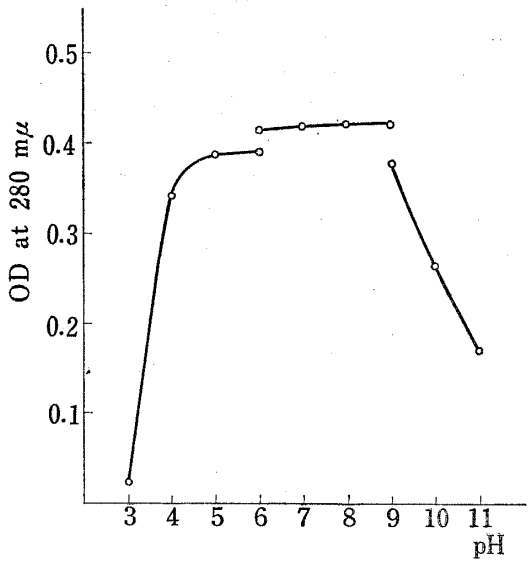

Fig. 3. PH Stability of Kinonase BI

1/10M citrate buffer at pH 3—6, 1/10M 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 1/10M Tris-HCl buffer (pH7.0) for 20 min at 37°.

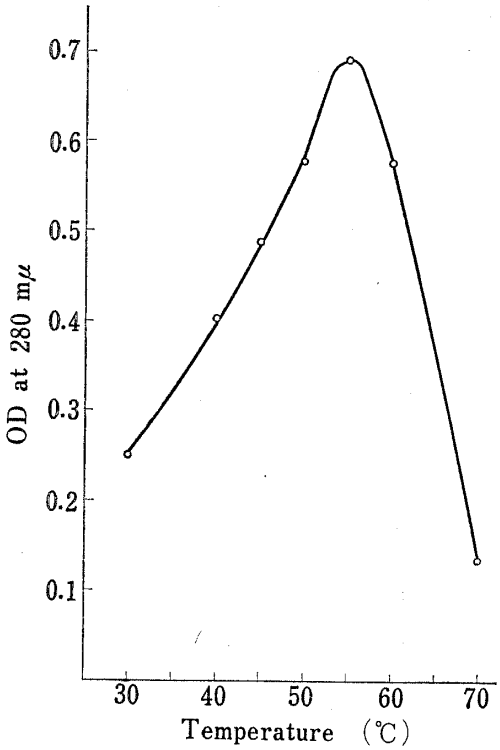

Fig. 4. Effect of Temperature on Proteolytic Activity of Kinonase BI

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

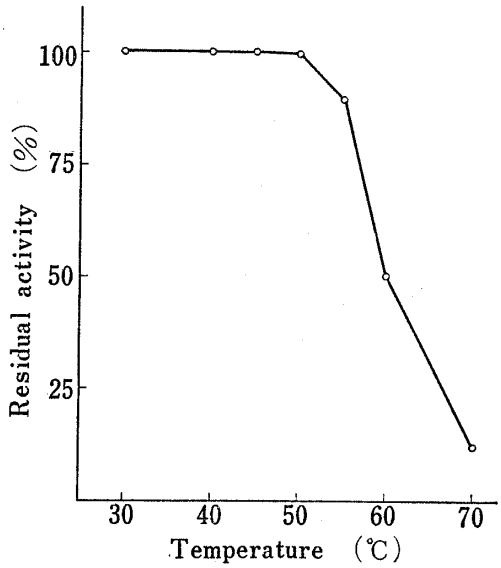

Fig. 5. Thermal Stability of Kinonase BI

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°.

analysis. Optimum pH of kinonase BI against casein is around 6.5 as shown in Figure 2, while that of kinonase AI or AIII is around 7.5. Kinonase BI is rather stable in a solution of pH 5—9, but not in alkaline and more unstable in acidic solutions (Figure 3). Kinonase BI shows the highest proteolytic activity at 55° when incubated with casein at pH 7.0 for 20 min as shown in Figure 4. The proteolytic activity of kinonase BI is stable in an aqueous solution at 50° for 10 min, while 50% of the activity is lost at 60° for 10 min as seen in Figure 5. Effects of various metal ions and chemicals are listed in Table I and Table II. The optimum pH of kinonase BI 6.5 is rather lower than those of neutral proteinases of Streptomyces origin. The proteolytic activity of kinonase BI is not inactivated when preserved with potato trypsin inhibitor, *p*-chloromercuribenzoic acid, *ω*-chloroacetophenone or diisopropylfluorophosphate, but inactivated by ethylenediaminetetraacetic acid. The substrate specificity of kinonase BI for various synthetic substrates is studied and the result is shown in Table III. Kinonase BI possess specificity toward phenylalanine, tyrosine and leucine and hydrolyzes the peptide

Table I. Effect of Metal Ions on the Activity of Kinonase BI

Metal ions	Residual activity (%) Kinonase BI	Metal ions	Residual activity (%) Kinonase BI
$AgNO_3$	83	Li_2SO_4	100
$CaCl_2$	95	$MgCl_2$	100
$CoCl_2$	87	$MnCl_2$	78
$CuSO_4$	8	$NaNO_2$	100
$FeSO_4$	55	$ZnSO_4$	59
$HgCl_2$	5		

Table II. Effect of Various Chemicals on the Activity of Kinonase BI

Chemicals	Residual activity (%) Kinonase BI	Chemicals	Residual activity (%) KinonaseBI
ω-Chloroacetophenone	100	Iodine	90
p-Chloromercuribenzoate	100	Glutathione	73
Monoiodoacetic acid	42	Glutathione-SSG	65
L-Cystine	114	N-Bromosuccinimide	2
Ethylenediaminetetraacetate	4	Cyanogen bromide	98
8-Hydroxyquinoline	100	Hydroxylamine hydrochloride	95
Diisopropylfluorophosphate	98	Potato trypsin inhibitor[a)]	100
Sodium lauryl sulfate	95	Potassium permanganate	17

effects of various inhibitors on proteolytic activity of kinonase BI
A mixture of 0.9 ml of the aqueous enzyme solution (6.7 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 (180 μg/ml) was used.

Table III. Substrate Specificities of Kinonase BI

Substrates	Kinonase BI	Substrates	Kinonase BI
D,L-Ala-Leu	—	N-Ac-D,L-Tyr-OEt	—
D,L-Ala-Gly-Gly	—	N-Ac-Tyr-OEt	—
Gly-Gly	—	N-Ac-D,L-Val	—
Gly-Leu	—	Cbz-Gly-Leu	—
Gly-Phe	—	Cbz-Gly-Phe-NH_2	+
Leu-Gly-Gly	—	Cbz-Gly-Pro-Leu-Gly	±
Leu-β-naphthylamide	—	Cbz-Glu-Phe	±
Ser-Tyr-Ser-Met	—	Cbz-Glu-Tyr	±
Ac-Gly	—	Cbz-Phe-Tyr	+
Ac-D,L-Met	—	Cbz-Trp-Leu-NH_2	+
N-Ac-D,L-Try	—		

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 1/400M 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 was applied to silicagel H thin-layer chromatography using BuOH:AcOH:H_2O=4:2:1. The digested substrate was ditected by splaying 1% ninhydrin solution.
+: hydrolyzed ±: weakly hydrolyzed —: not hydrolyzed

bond in which the amino groups of the above amino acid residues are involved. It is the same as kinonase AI and AIII. The mode of hydrolysis of kinonase BI on bradykinin is the same with that of kinonase AI and AIII. Bradykinin is hydrolyzed to arginylprolylprolylglycine, phenylalanylserylproline and phenylalanylarginine by kinonase BI.

Anti-inflammatory activity of kinonase BI is examined for carrageenin–induced edema in hind paw of rats. The activity is observed at the same level as kinonase AI and AIII.[3)] Kinonase BI showed 60% inhibition by intraperitoneal injection at a concentration of 5 mg/kg, while α-chymotrypsin showed 67% inhibition at a concentration of 100 mg/kg by the anti-inflammatory test.[3,6)]

Kinonase AII contaminated with a trace of kinonase AI hydrolyzes leucylglycylglycine, methylester of leucylphenylalanine and leucine-β-naphthylamide to give leucine, while the same substrates are not digested by kinonase AI. Thus, kinonase AII is presumed to be a kind of leucine amino peptidases. Kinonase AII does not show the anti-inflammatory activity

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

to carrageenin-induced edema in the hind paw of rats at a concentration of 5 mg/kg. Bradykinin is not also decomposed by kinonase AII.

Kinonase BI can be differentiated from all known neutral proteinases of Streptomyces origin as described below. The neutral proteinases of pronase,[7] R-F-1-a and F-2, have pH optima at 7.5—8 against casein. Ninety five per cent of the proteolytic activity of prozyme is destroyed by addition of diisopropylfluorophosphate.[8] The neutral proteinases of *Streptomyces fradiae* having the pH optima at 6.5—9, the fractions III and IV, retain more than 30% of their proteolytic activity by addition of 10^{-3}M of ethylenediaminetetraacetic acid.[9] The neutral proteinase of acidic protein nature from *Streptomyces naraensis* shows the maximum proteolytic activity at 40° against casein.

Experimental

Purification of Kinonase BI and AII——The crude mixture of kinonases[3] (15 g) obtained from the culture broth of *Streptomyces kinoluteus* was dissolved in H_2O (50 ml) and applied to a column of DEAE-cellulose (diameter 1.8 cm, high 45 cm). The column was eluted with aqueous NaCl (500 ml, pH 7.0) of a gradient concentration from 0.05M to 0.5M. The eluate was fractionated to each 20 ml and each fraction was dialyzed in a cellophane tube for 4 hr against distilled water containing a trace of $(AcO)_2Ca$ at 0°. Kinonase AI, AII and AIII were recovered from the fractions 5—15 and kinonase BI and AII were from the fractions 16—22. The mixture of kinonase BI and AII (538 mg), obtained as above, was again treated with a DEAE-cellulose column (diameter 1.6 cm, high 23 cm) and eluted with aqueous NaCl (500 ml, pH 7.0) of a gradient concentration from 0.05M to 0.5M. The eluate was fractionated to each 11.6 ml and the fractions No. 14—18 dialyzed as above gave a mixture of kinonase BI and AII (129 mg) by following lyophilization. The mixture of kinonase BI and AII (120 mg) was separated to each component on a column of Sephadex G-75 (diameter 1.8 cm, high 90 cm). The eluate was fractionated to each 10.0 ml and kinonase AII (21 mg) contaminated with a trace amount of kinonase AI was recovered from the fractions No. 6—8 by lyophilization, while kinonase BI (24 mg) from the fractions No. 10—12.

Hydrolysis of Kinonase BI with Constant boiling Hydrochloric Acid——Kinonase BI (3.0 mg) dissolved in constant boiling hydrochloric acid (2 ml) was hydrolyzed in a sealed tube at 110° for 17 hr. The hydrolyzate was evaporated to dryness and the residue was dried in a evacuated dessicator over NaOH for 17 hr. The amino acid analyzer, Model KLA-3B of Hitachi LTD, was used to determine the constituent amino acids of kinonase BI.

Hydrolysis of Bradykinin by Kinonase BI——The method described in the previous paper was used.[3]

Anti-inflammatory Effect of Kinonase BI and AII on Carrageenin-induced Edema——The method was described in the previous paper.[3]

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7) *a*) M. Nomoto and Y. Narahashi, *J. Biochem.*, **46**, 653, 839, 1481, 1645 (1959); *b*) M. Nomoto, Y. Narahashi and M. Murakami, **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.
8) Y. Yokote and Y. Noguchi, The Annual Meeting of Agricultural Chemical Society of Japan, Sapporo July 1964.
9) K. Morihara, T. Oka and H. Tsuzuki, *Biochim. Biophys. Acta*, **139**, 382 (1967).
10) A. Hiramatsu, *J. Biochem.*, **62**, 353, 364 (1967).