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Alkinonase A and AF, New Alkaline Proteinases produced by *Streptomyces violaceorectus*[1]

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Alkaline proteinases, named alkinonase A and AF, are isolated from the cultured broth of *Streptomyces violaceorectus*. Both alkinonases show remarkably strong anti-inflammatory activity for the carrageenin-induced edema. Alkinonase A and AF are belonging to a new type of alkaline proteinases of microbial origin. The effect of inhibitors on the proteolytic activity and the substrate specifities of alkinonases are resembled to that of the neutral proteinases of microbial origin and different from that of the alkaline proteinases of microbial origin. Production, isolation, purification, effect of pH on the proteolytic activity, pH stability, effect of temperature on the proteolytic activity, effect of inhibitors on the proteolytic activity, substrate specifities and anti-inflammatory activity for the carrageenin induced edema of alkinonases are described in this paper.

Kinonase AI, AIII, and BI produced by *Streptomyces kinoluteus*,[3,4] retikinonase I and II produced by *Streptomyces verticillatus* var. *zymogenes*,[5] a neutral proteinase produced by *Streptomyces griseolus*,[6] and neutral proteinase Fr. A and Fr. B produced by *Streptomyces cacaoi* var. *asoensis*[1] have been isolated as anti-inflammatory proteinases. Those enzymes are so-called microbial neutral proteinases having metalo-enzyme nature and show the similar enzymatic characters each other. Specially, the neutral proteinases have the substrate specificity to the peptide bond in which the amino group of phenylalanine, tyrosine or leucine is contained.[7] Even in a N-substituted dipeptide containes one of the above amino acids as the C-terminus, the proteinase usually hydrolyzes strongly or weakly the peptide bond to yield phenylalanine, tyrosine or leucine. Bradykinin is generally hydrolyzed to arginylprolylprolylglycine, phenylalanylserylproline and phenylalanylarginine by the neutral proteinases. Some of the above proteinases show remarkably strong anti-inflammatory activity for the carrageenin-induced edema of rat hind paw.

While alkaline proteinases of microbial origin are considered as serine enzymes.[7] and several alkaline proteinases have been isolated from the cultured filtrates of *Streptomyces fradiae*[8] and *Streptomyces griseus*.[9]

1) This is Part VI of "Anti-inflammatory Proteinases obtained from *Streptomyces*," by S. Nakamura. Part V: S. Nakamura, H. Fukuda, M. Hamada and H. Umezawa, *Chem. Pharm. Bull.* (Tokyo), **18**, 2581 (1970).
2) Location: *a*) *1-2-3, Kasumi, Hiroshima*; *b*) *3-14-23, Kamiosaki, Shinagawa-ku, Tokyo.*
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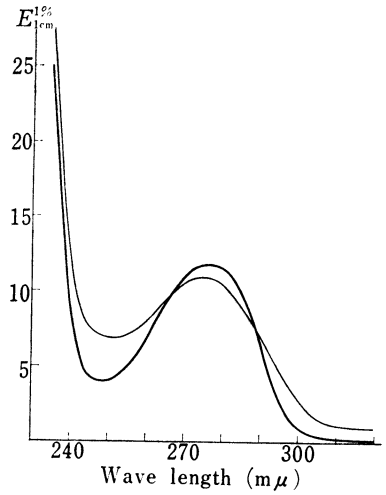

Fig. 1. Ultraviolet Absorption Spectra in H_2O

———: alinonase A
———: alkinonase AF

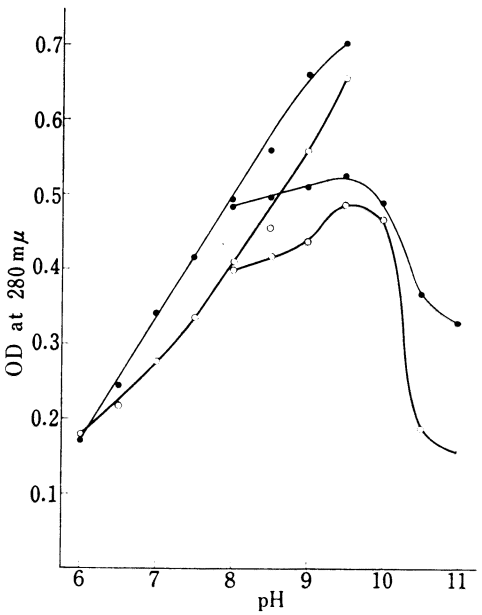

Fig. 2. Effect of pH on Proteolytic Activity

One ml of the aqueous enzyme solution (A=3 μg/ml, AF=6 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 8.5—11) for 20 min at 37°.

———: alkinonase A ———: alkinonase AF

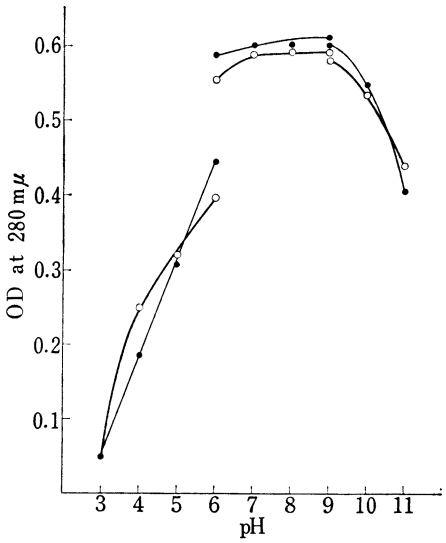

Fig. 3. pH Stability

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—11 were used. A mixture of 0.1 ml of the aqueous enzyme solution (A=30 μg/ml, AF=60 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/10 Tris-HCl buffer (pH 9.0) for 20 min at 37°.

———: alkinonase A ———: alkinonase AF

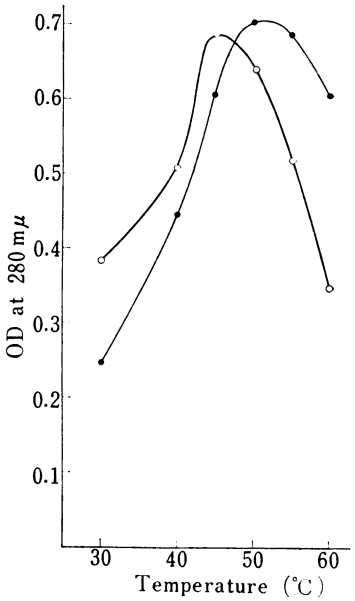

Fig. 4. Effect of Temperature on Proteolytic Activity

A mixture of 1 ml of the aqueous enzyme solution (A=1.5 μg/ml, AF=3 μg/ml) and 1% casein solution in M/10 Tris-HCl buffer (pH 9.0) was incubated at the test temperatures for 20 min.

———: alkinonase A ———: alkinonase AF

We have screened the cultured filtrates of *Streptomyces* for the alkaline proteinases to examine their enzymatic characters and anti-inflammatory activity. The alkaline proteinases, named alkinonase A and AF, have been isolated from the cultured broth of *Streptomyces* MC 675-A8 and the alkinonase producing strain is identified to be *Streptomyces violaceorectus*.[10] The enzymatic characters of both proteinases are shown to be out of the above category of alkaline proteinases of microbial origin.

Production, isolation, purification, enzymatic characteristics and anti-inflammatory activity of alkinonase A and AF are described in this paper.

Streptomyces violaceorectus was cultivated in a jar fermentor containing a medium composed of soy bean meal, potato starch, glucose and various metal salts to produce the alkaline proteinases at 27° for 69 hr. The crude proteinase mixture was recovered from the broth filtrate by saturation with ammonium sulfate, dialysis of the precipitate against water and following precipitation from the retantate by addition of acetone. The proteinase mixture was purified by gel filtration on Sephadex G 75 and further separated to two alkaline proteinase fractions by the gradient column chromatography on carboxy methyl cellulose (CMC). The earlier eluted proteinase by the gradient chromatography was named as alkinonase AF and the later was alkinonase A. The purity of both enzymes was tested by treatment with Ponceu 3R after electrophoresis on a cellulose acetate film (13 cm wide, 6 cm long) at 20 mA and 105 V for 40 min using 0.1M Tris-HCl buffer (pH 7.0). Alkinonase A and AF move 10 mm toward the cathode by the electrophoresis and can not be differentiated each other. Nevertheless, alkinonase AF is eluted earlier than the other by the gel filtration on Sephadex G 75 eluted with water. Each proteinase is finally purified by gel filtration on Sephadex G 75 to give the single spot at 10 mim toward the cathode by the electrophoresis. Thus, purified alkinonase A shows about twice proteolytic activity than purified alkinonase AF.

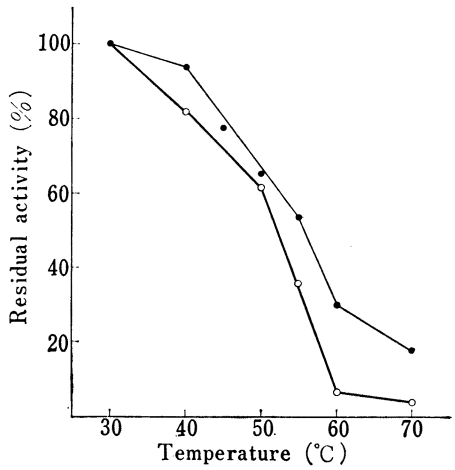

Fig. 5. Thermal Stability

One ml of the enzyme dissolved in M/100 Tris-HCl buffer (pH 9.0) (A=3 μg/ml, AF=6 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/10 Tris-HCl buffer (pH 9.0) for 20 min at 37°.

——: alkinonase A ——: alkinonase AF

The ultraviolet absorption spectra in water of alkinonase A and AF are shown in Fig. 1. The effect of pH on the proteolytic activity of both alkinonases against casein is illustrated in Fig. 2. The optimum pH of both proteinases is around 9.5 as seen in the figure. Alkinonase A and AF are stable at room temperature at pH 6—9 for 1 hr, but both proteinonses lose 90% of the proteolytic activity at room temperature at pH 3.0 for 1 hr as shown in Fig. 3. The effect of temperature on the proteolytic activity of alkinonases is shown in Fig. 4. The optimum temperature for alkinonase A is around 45° and that for alkinonase AF is around 50° when incubated with casein at pH 9.0 for 20 min. Both alkinonase are stable at 30° for 10 min in 0.1M Tris-HCl buffer (pH 9.0), but lose 30—35% of the proteolytic activity at 50° for 10 min in the same buffer as shown in Fig. 5. Various metal ions (10^{-3}M) and enzyme inhibitors (10^{-3}M) are added to examine the effect on the proteolytic activity of alkinonases

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Table I. Effect of Various Inhibitors on Proteolytic Activity

Metal ions	Residual activity (%) A	AF	Chemicals	Residual activity (%) A	AF
$CaCl_2$	87	80	*w*-chloroacetophenone	92	80
$CoCl_2$	160	145	*p*-chloromercuribenzoate	81	84
$CuSO_4$	54	51	monoiodoacetic acid	88	91
$FeSO_4$	99	104	L-cystine	74	77
$HgCl_2$	13	10	ethylenediaminetetraacetate	31	23
Li_2SO_4	87	92	8-hydroxyquinoline	33	36
$MgCl_2$	77	76	diisopropylfluorophosphate	94	99
$MnCl_2$	42	45	sodium laurylsulfate	76	80
$NaNO_2$	83	76	iodine	1	2
$ZnSO_4$	36	46	glutathione	73	68
			N-bromosuccinimide	4	5
			cyanogen bromide	65	77
			hydroxyamine hydrochoride	79	88
			potato trypsin inhibitor[a]	59	62
			soy bean trypsin inhibitor[a]	75	76

A mixture of 0.9 ml of the enzyme dissolved in M/10 Tris-HCl buffer (A=3 μg/ml, AF=6 μg/ml) (pH 9.0) and 0.1 ml of M/100 inhibitor solution was kept for 20 min at room temperature. Then, the mixture was incubated with 1 ml of 1% casein solution in M/10 Tris-HCl buffer (pH 9.0) for 20 min at 37°.
a) 0.1 ml of a solution (300 μg/ml) was used.

and the results are shown in Table I. Cobalt ion increases the activity of alkinonase A and AF, while mercuric ion reduces the activity of both enzymes. The proteolytic activity of the both alkaline proteinases is retained after addition of *w*-chloroacetophenone, *p*-chloromercuribenzoate or diisopropylfluorophosphate, but lost after addition of ethylenediamine tetraacetate. Substrate specificities of alkinonase A and AF for synthetic peptides are quite similar each other as seen in Table II. The specificities can be resembled to that of neutral proteinases of *Streptomyces* origin.

Table II. Substrate Specificities

Substrates	A	AF	Substrates	A	AF
D,L-Ala-Leu	—	—	Cbz-Glu-Phe	+	+
Gly-Gly	—	—	Cbz-Glu-Tyr	+	+
Gly-Leu	—	—	Cbz-Gly-Leu	+	+
Gly-Phe	—	—	Cbz-Gly-Phe	±	±
Ac-Gly	—	—	Cbz-Gly-Phe-NH_2	+	+
Ac-D,L-Met	—	—	Cbz-Gly-Pro-Leu-Gly	+	+
N-Ac-Try	—	—	Cbz-Phe-Tyr	+	+
N-Ac-D,L-Try-OEt	—	—	Cbz-Try-Leu-NH_2	+	+
N-Ac-Tyr-OEt	—	—			

All amino acids are L-form, unless otherwise specified.
A mixture of 1 ml of the aqueous enzyme solution (A=4 μg/ml, AF=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, ±: weakly hydrolyzed, —: not hydrolyzed

The anti-inflammatory activity of alkinonase A and AF was determined by intraperitoneal injection for the carrageenin-induced edema in the hind paw of rats.[11] Alkinonase A and AF have remarkably strong anti-inflammatory activity as shown in Table III. Specially

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

TABLE III. Inhibition of Carrageenin-Induced Edema in Hind Paw of the Rat by Intraperitoneal Injection

Drug	*i.p.* dose mg/kg	No. of rat	Body wietht gm±S.E.[a]	Edema, ml±S.E.[a] 1	3	5	24 hr	Inhibition of edma (%) 1	3	5	24 hr
Alkinonase A	0.8	5	142 ±2.4	0.16 ±0.019	0.31 ±0.036	0.39 ±0.019	0.50 ±0.047	30.4	67.0	60.6	47.4
	0.2	5	141 ±4.2	0.13 ±0.020	0.43 ±0.046	0.52 ±0.034	0.63 ±0.044	43.5	54.3	47.5	33.7
	0.05	5	140 ±2.2	0.18 ±0.020	0.64 ±0.026	0.76 ±0.024	0.73 ±0.086	21.7	31.9	23.2	23.2
	0.0125	5	144 ±3.9	0.22 ±0.012	0.65 ±0.022	0.77 ±0.020	0.75 ±0.014	4.3	30.8	22.2	21.1
Alkinonase AF	0.8	5	142 ±4.6	0.09 ±0.010	0.26 ±0.010	0.44 ±0.037	0.73 ±0.061	60.9	72.3	55.6	26.3
	0.2	5	142 ±3.5	0.15 ±0.035	0.41 ±0.045	0.60 ±0.042	0.76 ±0.043	34.8	56.4	39.4	20.0
	0.05	5	145 ±3.0	0.23 ±0.081	0.69 ±0.024	0.86 ±0.025	0.85 ±0.042	0	26.6	13.1	10.5
	0.0125	5	142 ±3.6	0.21 ±0.019	0.70 ±0.027	0.86 ±0.037	0.90 ±0.057	8.7	25.5	13.1	5.3
Control		5	143 ±2.6	0.28 ±0.012	0.94 ±0.037	0.99 ±0.043	0.95 ±0.014				

a) standard error

alkinonase A shows 30% inhibition of the edema by 0.0125 mg/kg at 3 hr after the injection of carrageenin.

The alkaline proteinases, peak III and peak IV, produced by *Streptomyces griseus*[9] and the alkaline proteinases, Fract, I_a, I_b, and II (keratinase), produced by *Streptomyces fradiae*[8] are known and considered to be serine enzymes as forementioned. The alkaline preteinases produced by *Streptomyces griseus* and *Streptomyces fradiae* retain their proteolytic activity after addition of ethylenediamine tetra-acetate, but lose their activity after addition of diisopropylfluorophosphate. The known alkaline proteinases of *Streptomyces* origin do not hydrolyze Z-Glu-Tyr. Thus, alkinonase A and AF can be differentiated from the above known alkaline proteinases of *Streptomyces* origin.

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] except pH value of 1% casein solution in M/10 Tris-HCl buffer was adjusted to 9.0.

Production of the Alkaline Proteinases——An inoculation seed was cultured on a reciprocal shaker (130 rpm) for 51 hr at 28° in 500 ml shaking flasks containing each 100 ml of a medium composed of 2% maltose, 0.5% peptone, 0.5% meat extract, 0.3% yeast extract, 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 7.4). A medium composed of 1.5% soy bean meal, 1% potato starch, 1% glucose, 0.3% NaCl, 0.1% K_2HPO_4, 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 7.6) was used to produce the alkaline proteinases. The inoculation seed (200 ml) was used to inoculate 12 liters of the production medium sterilized in a jar fermenter (25 liters) and fermented at 27° for 69 hr under aeration (10 liters/min) and stirring (250 rpm). Forty ml of silicon oil was added as an antifoaming agent to the jar fermenter.

Isolation of the Crude Alkaline Proteinases——The broth filtrate (11 liters) (pH 7.5) was suturated with $(NH_4)_2SO_4$ (6 kg) at 0° adjusting to pH 7.0 by addition of 1N NH_4OH to precipitate the proteinases. The precipitated enzyme mixture was collected by centrifugation and dialyzed in a cellophane tube against distilled water for 30 min at 0°. Cold acetone (480 ml) was added to the retantate (160 ml) and the precipitated enzyme mixture was collected and dried. Thus, 35.5 g of the crude enzyme was recovered from the fermentation broth recovering 50% of the proteolytic activity.

Purification of the Alkaline Proteinases——The crude alkaline proteinase mixture (10 g) dissolved in 50 ml of H_2O was purified by gel filtration on a column of Sephadex G 75 (71 cm × 3.5 cm diameter) eluted with H_2O. The eluate was collected in 17 ml fractions and the proteolytic activity at pH 9.5 and pH 7.0 of each fraction was determined. The alkaline proteinase fractions were recovered by lyophilization from fractions 15—25 (440 mg). The alkaline proteinase fractions showed 5 spots (7 mm, 8.5 mm, 10 mm, 12 mm and 17 mm toward the cathode) by the electrophoresis. The alkaline proteinase fractions (440 mg) dissolved in H_2O (100 ml) was applied to a column of CMC (37 cm × 2 cm diameter) (NH^+_4-type treated with 0.1M Tris-HCl buffer of pH 7.0) to separate the two alkaline proteinases. The column was eluted with a linear gradient of aqueous NaCl from 0 to 0.3M (total 800 ml) and collected in 9.0 ml fractions. The alkaline proteinase AF was eluted in fractions 10—13 (143 mg) and the alkaline proteinase A was from fractions 18—29 (220 mg) and the active fractions were separately lyophilized after dialysis in cellophane tubes for 2 hrs against distilled water at 0°. The alkaline proteinase AF (143 mg) was dissolved in H_2O (5 ml) and purified on a column of Sephadex G 75 (78 cm × 2.2 cm diameter) eluted with H_2O. The eluate was fractionated to each 10 ml of fraction and the alkaline proteinase AF was eluted in fractions 12 (7.5 mg), 13 (6.6 mg) and 14 (7.0 mg) showing one spot at 10 mm toward the cathode by the electrophoresis. While three spots at 7 mm, 8.5 mm and 10 mm to the same direction were detected from the fractions 16—20 showing the proteolytic activity. The fractions 18—29 (210 mg) was also purified on a column of Sephadex G 75 (78 cm × 2.2 cm diameter) and fractionated to each 10 ml of fraction. The alkaline proteinase A was recovered from the fractions 23 (13 mg), 24 (15 mg), 25 (10 mg) and 26 (7 mg) by lyophilization. The proteinase recovered from fractions 23 and 24 showed the main spot at 10 mm and a minor spot at 8.5 mm toward the cathode by the electrophoresis, while fractions 25 and 26 showed one spot at 10 mm to the same direction.

Assay of the Anti-inflammatory Activity of Alkinonase A and AF for Carrageenin-induced Edema——Young adult male Wistar rats of body weight 130—150 g were employed. 0.1 ml of 1% carrageenin in 0.9% saline was injected subcutaneously into the plantar aponeurosis of one hind paw of each of the 5 rats at 1 hr after intraperitoneal administration of the enzyme solution. 0.1 ml of 0.9% saline was also injected to the contralateral paw as the control. Swelling of the paw reached to the maximum in 3 to 5 hr, then retained the almost same degree of edema for several hrs. The increase in size of the carrageenin-injected paw over the control paw was determined by fluid displacement after 1, 3, 5, and 24 hr. Anti-inflammatory activity of the enzymes was determined by computation of the per cent inhibition using the mean paw size of the drug-treated group as compared with that of the control group.