[Chem. Pharm. Bull. 18(10)2112—2118(1970)]

UDC 615.276.011.4 : 615.332.011.4

Anti-inflammatory Neutral Proteinases, Retikinonase I and II obtained from *Streptomyces verticillatus* var. *zynogenes*[1)]

Shoshiro Nakamura,[2a)] Masa Hamada, Masaaki Ishizuka and Hamao Umezawa[2b)]

Pharmaceutical Institute, Hiroshima University School of Medicine[2a)] *and Institute of Microbial Chemistry*[2b)]

(Received May 13, 1970)

New neutral proteinases, designated retikinonase I and II, have been isolated from the cultured broth of a Streptomyces strain. Both enzymes are purified by gradient column chromatography on DEAE-cellulose and gel filtration on a column of Sephadex G-75. Retikinonase I and II are of basic protein nature and contain the same constituent amino acids. Both enzymes have enzymatic characteristics similar to neutral proteinases of microbial origin and hydrolyze bradykinin by the same manner with kinonase AI, AIII or BI. Retikinonase I showed very high anti-inflammatory activity for carrageenin-induced edema in the hind paw of rats, but retikinonase II did not show the significant activity.

The retikinonase-producing strain is considered to be similar to *Str. uerticillatus*.

Taxonomic studies of the producing organism, production, isolation, purification, pysicochemical properties, effect of pH on proteolytic activity, pH stability, effect of temperature on proteolytic activity, thermal stability, effect of inhibitors on proteolytic activity, andsubstrate specificities of retikinonase I and II are reported in this paper.

The culture filtrates of 1300 microbes were tested for antibradykinin activity and eleven *Streptomyces* strains showed significant antagonistic activity to bradykinin.[3)] The active components of these strains were found to be proteinases. From *Streptomyces kinoluteus*, we isolated the new neutral proteinases, kinonase AI, AIII and BI.[3,4)] These materials have anti-inflammatory activity when tested against carrageenin-induced edema of the rat paw.

Two neutral proteinases named retikinonase I and II respectively have been isolated from the cultured broth of *Streptomyces* strains MA 603—A2 designated as *Streptomyces verticillatus* var. *zymogenes*. Both retikinonases have been differentiated from known neutral proteinases produced by *Streptomyces* including *Str. griseus*,[5)] *Str. fradiae*,[6)] *Str. caespitosus*,[7)] and *Str. naraensis*.[8)] Strong proteolytic activity has also been observed in the cultured broths of *Str. albus*,[9)] *Str. phaeochromogenes*,[7)] and *Str. ambofaciens*.[7)] Retikinonase I and II have

1) This is Part III of "Anti-inflammatory Proteinases obtained from *Streptomyces*" by S. Nakamura. Part II: S. Nakamura, Y. Marumoto, H. Miyata, I. Tsukada, N. Tanaka, M. Ishizuka and H. Umezawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 2044 (1969).
2) Location: *a*) *1-2-3, Kasumi-cho, Hiroshima*; *b*) *3-14-23, Kamiosaki, Shinagawa-ku, Tokyo.*
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).
4) S. Nakamura, Y. Marumoto, H. Miyata, I. Tsukada, N. Tanaka, M. Ishizuka and H. Umezawa, *Chem Pharm. Bull.* (Tokyo), **17**, 2044 (1969).
5) *a*) A. Hiramatsu and T. Ouchi, *J. Biochem.*, **54**, 462 (1963); *b*) Y. Narahashi and M. Yanagita, *J Biochem.*, **62**, 633 (1967); *c*) Y. Narahashi, K. Shibuya and M. Yanagita, *J. Biochem.*, **64**, 427 (1968)
6) *a*) J.J. Noval and W.J. Nickerson, *J. Bacteriol.*, **77**, 251 (1959); *b*) W.J. Nickerson and J.J. Noval, *Biochim. Biophys. Acta*, **77**, 73 (1963); *c*) W.J. Nickerson and S.C. Durand, *Biochim. Biophys. Acta*, **77**, 87 (1963); *d*) K. Morihara, T. Oka and H. Tsuzuki, *Biochim. Biophys. Acta*, **139**, 382 (1967).
7) *a*) Y. Yokote, K. Kawasaki, J. Nakajima and Y. Noguchi, *J. Agr. Chem. Soc. Japan*, **43**, 125 (1969); *b*) Y. Yokote and Y. Noguchi, *J. Agr. Chem. Soc. Japan*, **43**, 132 (1969).
8) A. Hiramatsu, *J. Biochem.*, **62**, 353, 364 (1967).
9) D.J. Hirschman, J.M. Zametkin and R.E. Rogers, *Am. Dyestuff Reptr.*, **33**, 353 (1944).

enzymatic characteristics similar to other neutral proteinases of microbial origin[10] and both retikinonases hydrolyze bradykinin to arginylprolylprolylglycine, phenylalanylserylproline and phenylalanylarginine. The hydrolysis of bradykinin by both retikinonases resembles that of kinonase AI, AIII and BI but is different from that of α-chymotrypsin, carboxypeptidase B, carboxypeptidase N, proteinase b and kinonase II, known bradykinin-hydrolyzing enzymes.[3,4,11] Retikinonase I shows a strong anti-inflammatory activity on carrageenin-induced edema but retikinonase II dose not.

The retikinonase-producing *Streptomyces* was isolated from a soil sample collected at Higashikurume-machi, Kitatama-gun, Tokyo in November 1965. On microscopic examination, long and straight hyphae are observed extending from fine branched basic hyphae with primary and secondary whorls. No spiral formation is seen. The spore surface is smooth. The cultural characteristics on various media are shown in Table I.

Table I. Cultural Characteristics of *Str. verticillatus* var. *zymogenes* on Various Media

	Growth	Aerial mycelium	Soluble pigment	Comments
Glycerol nitrate agar, 27°	colorless to pale brown	white to yellowish white	none	
Glucose-asparagine agar, 27°	colorless	at first thin white but later light olive gray, abundant, cottony	none	
Calcium malate agar, 27°	colorless	white to grayish white but later light olive gray	none	dose not dissolve calcium malate
Peptone solution (containing 1.0% of $NaNO_3$), 27°	colorless	scant white	faint brown to dark brownish	no nitrate reduction
Starch agar, 27°	colorless to pale brown	white to light olive gray	none	starch hydrolysis is observed
Tyrosine agar, 27°	colorless	white	black	tyrosinase is positive
Potato plug, 27°	pale brown	white to light brownish gray to light gray	brownish black	
Nutrient agar 27°C or 37°	colorless to pale brown	thin white	brown	
Loeffler's serum 37°	wrinkled, colorless to pale brown	none	dark brown at circumference	liquefaction not observed
Gelatin, 20°	colorless to pale brown	white	light brown to brown to dark brown	no liquefcation
Skimmed milk 37°	dark brown to brownish black	none	dark brown	strong peptonization occurs after coagulation of milk
Cellulose, 27°	colorless	none	none	dose not decompose

The culture was checked for utilization of carbon sources in Pridham-Gottlieb basal medium. It grows well utilizing starch, dextrin, glycerol, glucose, maltose, and mannose. It is difficult to determine whether fructose is utilized or not. Saccharose, inositol, lactose, mannitol, rhamnose, inulin, sorbitol, dulcitol, xylose, arabinose, and salicin are not utilized. As shown in Table I, strain MA 603-A2 belongs to the genus *Streptomyces*, and its characteristics can be summarized as follows: Whorl formation; no spiral; smooth structure on surface of spore; colorless to pale brown growth; while to light olive grey aerial mycelium; chromogenic type. This strain resembles *Streptomyces griseus* in growth on various kinds of media

10) *a*) K. Morihara, *Biochim. Biophys. Res. Commun.*, **26**, 656 (1957); *b*) K. Morihara, H. Tsuzuki and T. Oka, *Arch. Biochem. Biophys.*, **123**, 572 (1968).
11) H.Y.T. Yang and E.G. Erdös, *Nature*, **215**, 1402 (1967).

and the superficial appearance of aerial hyphae, but is different since the hyphae of MA 603–A2 show whorl formation and are the chromogenic type.

Streptomyces verticillatus[12] is considered to be the most similar species as shown in Table II.

Table II. Comparison MA603-A2 and *Str. verticillatus*

Spore surface	MA603-A2 smooth	*Str. verticillatus* smooth
Whorl formation	+	+
Color of aerial mycelium	white to light olive grey	white to dark grey or greyish green
Melanin	+	+
Liquefaction of gelatin	−	quick
Coagulation and peptonization of milk	+	+
Hydrolysis of starch	+	+
Nitrate reduction	−	quick
Antibiotics produced	weak	weak

As both strains are very similar except for the liquefaction of gelatin and nitrae reduction, they are not considered different species.

The retikinonase producing strain was cultivated in a medium containing glucose, starch, soy bean meal, and various metal ions in a jar fermentor to produce retikinonases. Proteolytic activity of the enzymes was measured by the method used for kinonases.[3] A proteinase mixture was precipitated from the cultured filtrate by addition of ammonium sulfate. The precipitate was lyophilized after dialysis against water to yield crude powder of retikinonases. The crude retikinonase mixture was separated to two portions having proteolytic activity by DEAE-cellulose column chromatography with gradient elution of a saline. The first eluted proteolytic portion was further separated to two proteinase fractions by gel filtration on a Sephadex G 75 column. The proteinase recovered from the earlier eluate of gel filtration was named retikinonase I and the later was retikinonase II. The later eluted proteinase by DEAE-cellulose column chromatography is not yet purified but consequentally named retikinonase III. The purity of each enzyme was examined by electrophoresis and by enzymatic studies of the effect of pH, substrate specifity *etc.* Retikinonase I can not be differentiated from retikinonase II by electrophoresis since both move 0.7 cm toward the cathode after electrophoresis at 8 mA and 75 V on Separax (cellulose acetate film, 8 cm wide, 6 cm long) for 1 hr using 0.01 M phosphate buffer solution (pH 7.0) containing 0.1 M sodium chloride. The amino acid analysis of the hydrolyzate of retikinonase I or II with constant boiling hydrochloric acid at 110° for 20 hrs in a sealed tube gave Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Met, Leu, Ile, Tyr, Phe, Lys, His, NH_3, Arg, and small amount of Cys by the Stein and Moore method.

The ultraviolet absorption maxima of retikinonase I and II in water are at 276—278 mμ as shown in Fig. 1. The effect of pH on the proteolytic activity of retikinonase I and II against casein is illustrated in Fig. 2. The optimum pH of retikinonase I is around 7.5—8.0, while that of retikinonase II is 7.0—7.5. As shown in Fig. 3, retikinonase I is rather stable at room temperature at pH 6—9 for 1 hr, but more than 20% of the proteolytic activity of retikinonase II is lost under these condition. Most of the activity of both proteinases is lost within 1 hr in a solution at pH less than 4 or more than 10. The effect of temperature on proteolytic activity of retikinonases is shown in Fig. 4. The optimum temperature for both enzymes is around 55° when incubated with casein at pH 7.0 for 20 min. Thermal satability of retikino-

12) S.A. Waksman, *J. Bacteriol.*, **81**, 70 (1961).

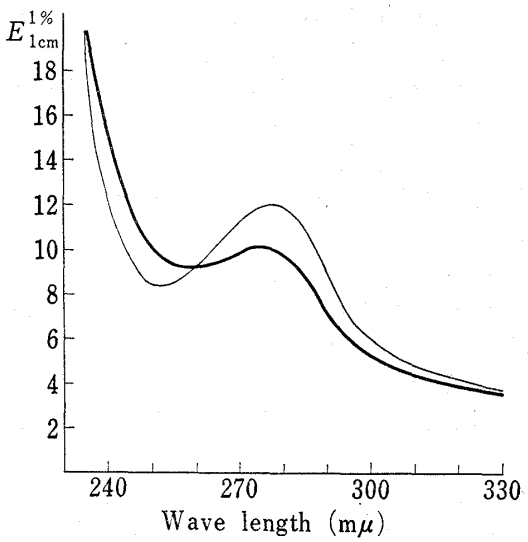

Fig. 1. UV Spectra of Retikinonases in H_2O

retikinonase I : ——
retikinonase II: ——

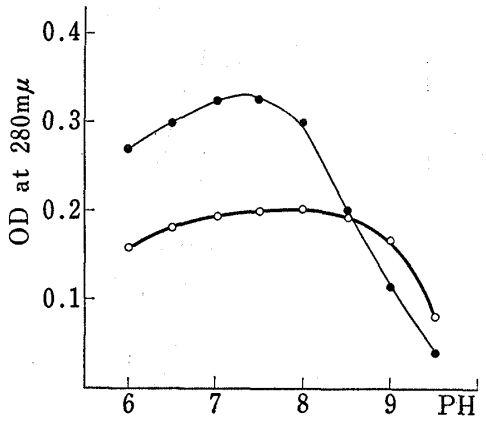

Fig. 2. Effect of pH on Proteolytic Activity of Retikinonases

One ml of the aqueous enzyme solution (5 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°.

retikinonase I : —○—
retikinonase II: —●—

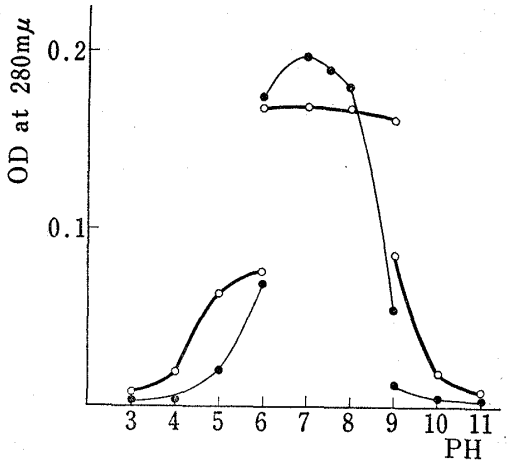

Fig. 3. pH Stability of Retikinonases

1/10M citrate buffer at pH 3—6, 1/10M Tris-HCl buffer at pH 6—9 and 1/10M borate buffer at pH 9—11 were used. A mixture of 0.1 ml of the aqueous enzyme solution (5 μg/ml) and 0.2 ml of the buffer was kept for 1 hr at room temperature. 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 1/10M Tris-HCl buffer (pH 7.0) for 20 min at 37°.

retikinonase I: —○—
retikinonase II: —●—

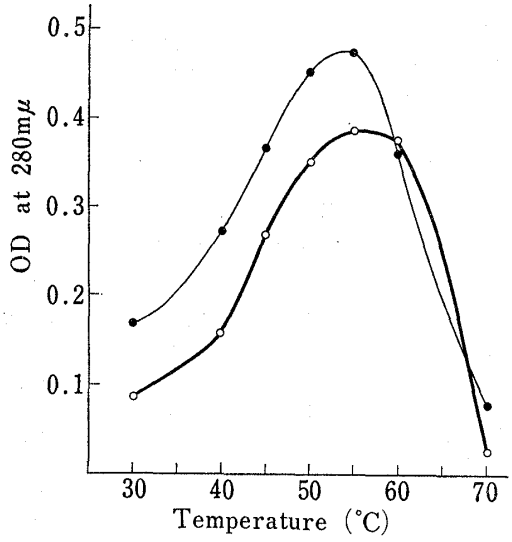

Fig. 4. Effect of Temperature on Proteolytic Activity of Retikinonases

A mixture of 1 ml of the aqueous enzyme solution (5 μg/ml) and 1 ml of 1% casein solution in 1/10M Tris-HCl buffer (pH 7.0) was incubated at the test temperature for 20 min.

retikinonase I: —○—
retikinonase II: —●—

nase I and II in presence or absence of calcium ion is shown in Fig. 5. Retikinonase I is stable at 50° for 10 min in 0.1M Tris-HCl buffer (pH 7.0), but retikinonase II loses activity under these conditions. Calcium ion has a protective action for both proteinases. The effect of various metal ions and chemicals on the proteolytic activity of retikinonases are shown in Table III. With retikinonase I and II, calcium, lithium or magnesium ion does not reduce proteolytic activity, but most of the activity of both enzymes is lost in the presence of mercuric or cupric ion. The enzymatic activity of both proteinases is retained after addition of ω-chloroacetophenone, *p*-chloromercuribenzoate, diisopropylfluorophosphate or potato trypsin

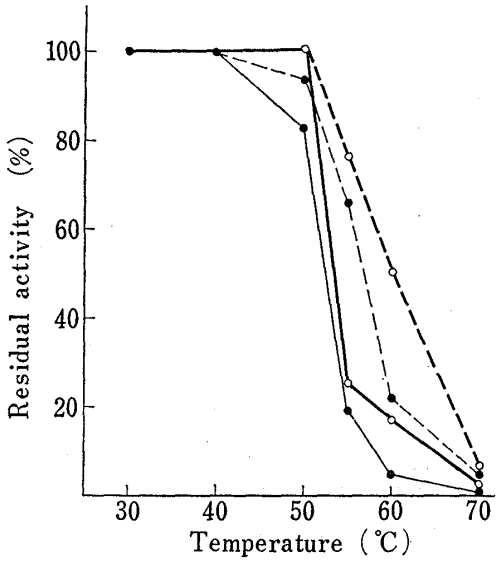

Fig. 5. Thermal Stability of Retikinonases

One ml of the enzyme dissolved in 1/10M Tris-HCl buffer (pH 7.0) (5 μg/ml) was heated at test temperature for 10 min. After cooling, the resulting solution was incubated with 1 ml of 1% casein solution in 1/10M Tris-HCl buffer (pH 7.0) for 20 min at 37°.

	added	1/1000M $(AcO)_2Ca$
retikinonase I:	—○—	---○---
retikinonase II:	—●—	---●---

inhibitor, but lost after addition of ethylenediamine tetra-acetic acid. Substrate specificities of retikinonase I and II are shown in Table IV. Both enzymes have specificity toward phenylalanine, tyrosine and leucine and hydrolyze the peptide bond in which the amino group of above amino acid residues is contained. Retikinonase I or II easily hydrolyzes even the peptide bond of a dipeptide when one of the above amino acid is involved as C-terminus in the form of an amide and the N-terminal amino group is substituted. Bradykinin is hydrolyzed to arginylprolylprolylglycine, phenylalanylserylproline and phenylalanylarginine by retikinonase I or II. The same results were obtained with kinonase AI, AIII or BI.[3,4]

The anti-inflammatory activity of retikinonase I and II was tested with carrageenin-induced edema in the hind paw of rats.[3,13] Retikinonase I showed much higher anti-inflammatory activity than kinonases,[3,4] but retikinonase II did not show significant anti-inflammatory activity. Retikinonase I showed 70% inhibition of the edema by intraperitoneal injection of 5 mg/kg or 1.25 mg/kg and 54% inhibition with 0.31 mg/kg, while retikinonase II have 6% and 4% inhibition at 2.5 mg/kg and 0.62 mg/kg. α-Chymotrypsin showed 65% inhibition at 100 mg/kg in the test.

Table III. Effects of Various Inhibitors on Proteolytic Activity of Retikinonases

Metal ions	Residual activity (%) Retikinonase I	Retikinonase II	Chemicals	Residual activity (%) Retikinonase I	Retikinonase II
$AgNO_3$	18	32	ω-chloroacetophenone	100	100
$CaCl_2$	99	100	*p*-chloromercuribenzoate	89	92
$CoCl_2$	64	57	monoiodoacetic acid	47	23
$CuSO_4$	4	3	L-cystine	97	100
$FeSO_4$	58	38	ethylenediamine tetra-acetic acid	0	0
$HgCl_2$	2	0	8-hydroxyquinoline	100	88
Li_2SO_4	98	100	diisopropylfluorophosphate	92	86
$MgCl_2$	100	94	sodium laurylsulfate	95	77
$MnCl_2$	86	91	iodine	0	0
$NaNO_2$	92	97	glutathione	65	28
$ZnSO_4$	47	42	glutathione-SSG	62	38
			N-bromosuccinimide	50	31
			cyanogen bromide	94	96
			hydroxylamine hydrochloride	94	75
			potato trypsin inhibitor[a]	100	97
			potassium permanganate	2	0

A mixture of 0.9 ml of the aqueous enzyme solution (8 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.

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

Table IV. Substrate Specificities of Retikinonases

Substrates	Retikinonase I	Retikinonase II	Substrates	Retikinonase I	Retikinonase II
D,L-Ala-Gly-Gly	—	—	Cbz-Glu-Phe	—	—
D,L-Ala-Leu	—	—	Cbz-Gly-Leu	—	—
Gly-Gly	—	—	Cbz-Gly-Phe	—	—
Gly-Leu	—	—	Cbz-Gly-Phe-NH_2	+	+
Gly-Phe	—	—	Cbz-Gly-Pro-Leu-Gly	—	—
Gly-Phe-NH_2	—	—	Cbz-Phe-Tyr	—	—
Leu-Leu	—	—	Cbz-Try-Leu-NH_2	+	+
Ac-D,L-Met	—	—	Z-Gly-Leu-NH_2	+	+
N-Ac-D,L-Try	—	—	Z-Gly-Pro-Leu	—	—
N-Ac-D,L-Try-OEt	—	—	Z-Pro-Leu-NH_2	+	—
Ac-D,L-Val	—	—			

All amino acids are L-,unless otherwise specified.
A mixture of 1 ml of the aqueous enzyme solution (12 μg/ml) and 1 ml of 1/400M substrate solution in 1/100M Tris-HCl buffer (pH 7.0) was incubated for 20 hr at 37° and lyophilized. The residue was dissolved in 0.1 ml of 50% aqueous methanol and chromatographed on silica gel H using BuOH:AcOH:H_2O=(4:2:1). The digestion products was detected by spraying 1% ninhydrin solution.
+: hydrolyzed, −: not hydrolyzed

Kinonase AI and AIII move 1.5 cm toward the cathode and retikinonase I and II move 0.7 cm in the same direction after electrophoresis at 8 mA and 75 V for 1 hr on Separax using 0.01M phosphate buffer (pH 7.0) containing 0.1M sodium chloride.[3,4] Pronase produced by *Str. griseus* K-1 is known to contain at least four neutral proteinases, two of which R-1-d and peak II, have been isolated. Those two neutral proteinases from pronase are rather stable at pH 5—9. More than 89% of their activity is retained after heating for 10 min.[5] The neutral proteinases produced by *Str. fradiae,* fraction III and IV, show maximum proteolytic activity at pH 6.5—9 and more than 30% of their proteolytic activity is retained in the presence of ethylenediamine tetra-acetic acid.[6] Ninety-five per cent of the proteolytic activity of the neutral proteinase from *Str. caespitosus* is lost on addition of diisopropylfluorophosphate.[7] The neutral proteinase produced by *Str. naraensis* is acidic and shows maximum proreolytic activity at 40°.[8] Thus, retikinonase I and II can be differentiated from the above known neutral proteinases from Streptomyces.

Experimental

Production of Retikinonases——A medium composed of 1% Prorich (soy bean meal produced by 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% $CuSO_4 \cdot 7H_2O$ (pH 7.4) was used for preparation of the inoculation seed and production of retikinonases. The inoculation seed was cultured in a 500 ml shaking flask containing 100 ml of the medium at 28° for 48 hr. The cultured seed (400 ml) was used to inoculate 20 liters of the same medium sterilized in a stainless jar fermentor (30 liters) and fermented at 27° for 68 hr under aeration (20 liters/min) and stirring (300 rpm). Sterilized soy bean oil was added to the fermentation broth as an antifoaming agent during the fermentation.

Isolation of Crude Retikinonases——The mycelium cake was removed by filtration and $(NH_4)_2SO_4$ (7 kg) was added to the broth filtrate (15.5 liters) at 5° and adjusted to pH 7 to precipitate the enzymes. The precipitated crude enzyme mixture was recovered by cold centrifugation and dialyzed in a cellophane tube against water containing 10^{-3}M $(AcO)_2Ca$ for 16 hr. Insoluble material in the retentate was removed and the retentate was lyophilized to yield a crude retikinonase mixture (13.5 g). Thus, 53% of the proteolytic activity of the cultured broth was recovered by the above treatment.

Purification of Retikinonase I and II——The crude retikinonase mixture (3.5 g) was dissolved in 100 ml of H_2O and purified on a column of DEAE-cellulose (25 cm × 2 cm diam.) adjusted to pH 7.0 by treatment with 1N HCl and 0.1M Tris-HCl buffer (pH 7.0) at 5°. The column was eluted with a linear gradient of aqueous NaCl from 0.05 to 0.5M (total 600 ml) and the eluate was collected in 16.0 ml fractions. The first peak of the proteolytic activity was found in fractions 7—11 and the second in fractions 13—16. The active fractions were separately dialyzed in cellophane tubes for 3 hr at 0° against distilled water containing a

trace of $(AcO)_2Ca$ and lyophilized. Thus, a white powder containing retikinonase I and II (844 mg) was recovered from fractions 7—11 and a mixture containing retikinonase III (468 mg) from fractions 13—17. The mixture of retikinonase I and II (150 mg) was dissolved in H_2O (5 ml) and separated by gel filtration on a column of Sephadex G 75 (90 cm × 1.8 cm diam.). The eluate was collected in 11.6 ml fractions. Retikinonase I (23 mg) was recovered from fractions 6—7 and retikinonase II from fractions 14—15 (18 mg) by lyophilization.