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Partial Purification of Ribonuclease from *Trichoderma koningii*

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Three ribonucleases (RNases) were partially purified from *Trichoderma koningii* by means of ammonium sulfate fractionation, column chromatographies on Amberlite IRC-50, Sephadex G-100, phospho-cellulose or diethylaminoethyl (DEAE)-cellulose and Sephadex G-75. Three RNases fractions, fraction I—III, are non-specific in respect of bases. Fraction I and II released nucleotides from ribonucleic acid (RNA) in the order of A>U>G>C and fraction III released nucleotides in the order of G>A>U>C. Molecular weight of three fractions estimated by gel filtration was 24000, 18000 and 6000, respectively. pH optimum of fractions I and II was at pH 4.3 and that of fraction III was at pH 4.8. Cu^{2+}, Zn^{2+} and Hg^{2+} inhibited fractions I and II at the concentration of 10^{-3}M, but fraction II moderately. Mg^{2+} had no effect on the enzymatic activity of fraction I and II, but it accelerated fraction III activity considerably.

Since ribonucleases (RNases) which have definite base specificity are very important tools for the structural studies of ribonucleic acid (RNA), many attempts have been carried out to find new RNases from microorganisms, such as fungi and bacteria, and a lot of useful enzymes were isolated as pure form.[2,3] However, for RNA chemistry and enzymology of RNase, it is yet requested to search new enzymes which have a new specificity.

In this report, we described the partial purification of RNases from a kind of fungi, *Trichoderma koningii* and some of their properties. One of the RNases was found to be eluted from Bio-Gel P-10 column between cytochrome c and insulin and the molecular weight of this enzyme estimated by gel filtration was about 6000. The molecular weight of other two enzymes were about 24000 and 18000. The three RNases were non-base specific enzymes.

Experimental

Enzyme Source——A commercial digestive "Meicelase P" (*Trichoderma koningii*) was used for RNase source.

Substrate——RNA was purchased from Kojin Co., Ltd., and used without further purification.

Enzyme Assay——The RNase activity during purification was based on the hydrolysis of RNA.[4] In a routine assay (standard assay), the reaction mixture (2 ml) contained 5 mg RNA, 50 mM acetate buffer (pH 5.0) and enzyme. The mixture was incubated at 37° for 5 min, after which 1 ml of the MacFadyen reagent[5] was added to stop the reaction. The precipitate formed was removed by centrifugation and 0.3 ml of the supernatant was diluted with 2 ml of water, and the absorbancy at 260 nm was determined. A unit of activity corresponds to the amount of enzyme necessary to cause an absorbancy change of 1.0 at 260 nm per 5 min in the assay conditions described above. The desoxyribonucleases (DNase) activity was also measured in the presence of Mg^{2+} (10 mM) using the above mentioned reaction conditions. DNase, phosphomono- and di-esterase activity were measured as reported previously.[4]

Protein Determination——Protein concentration was measured on the bases of absorbancy at 280 nm of 0.1% solution was 1.0.

1) Location: *a) Sakyo-ku, Kyoto; b) Ebara 2-4-41, Shinagawa-ku, Tokyo.*
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Hydrolysis of RNA by RNase Fractions——Four mg of RNA dissolved in 300 μl of acetate buffer (0.14M, pH 5.0) and 2 units of each enzyme were incubated at 30°. The 50 μl aliquots were withdrawn at appropriate time intervals, and chromatographed two dimensionally by ascending fashion using solvent systems, iso-butyric acid: 0.5N NH_4OH (10: 6 v/v) (the first dimension) and iso-propanol: ammonium sulfate saturated water: 0.1M citrate–phosphate buffer (pH 6.0) (2: 80: 20 v/v) (the second dimension). The optical densities of the mononucleotide spots were measured at 260 nm in 0.1N HCl after 18 hr extraction.

Results

Purification

Two hundred grams of a commercial digestive "Meicelase P" (*Trichoderma koningii*) (Meiji Seika Co., Ltd.) was dissolved in 600 ml of water. The solution was centrifuged for 10 min at 4000 rpm. The pH of the supernatant was adjusted to 4.7 by addition of 4N NaOH and the solution was brought to 30% saturation of ammonium sulfate by adding solid ammonium sulfate, and centrifuged for 15 min at 8000 rpm. The supernatant was brought to 85% saturation of ammonium sulfate, and kept at 4° overnight. The precipitate formed was collected by centrifugation and dissolved in 200 ml of distilled water and the resulting solution was dialyzed against distilled water.

IRC-50 Column Chromatography

The dialyzed solution was adjusted to pH 3.5 by addition of glacial acetic acid and then applied on an IRC-50 column. Three enzymatic active fractions appeared (Fig. 1); fraction I which was eluted first and fraction III which was eluted last and activated by Mg^{2+}, and fraction II which was eluted between fraction I and III. Each fraction was rechromatographed on an IRC-50 column using the same conditions as Fig. 1. and the enzymatic active fractions were pooled.

Sephadex G-100 Column Chromatography

Fraction I, II and III were chromatographed on Sephadex G-100. Fractions I, II and III were eluted at tube No. 106, 114 and 116, respectively (Fig. 2).

Diethylaminoethyl (DEAE)-Cellulose Column Chromatography

Fractions I and II eluted from Sephadex G-100 were adjusted to pH 8.5 by addition of 4N NaOH and applied on a column of DEAE–cellulose, separately. Although fraction I was eluted as a single enzymatic active peak, fraction II was found to contain three fractions. Since the faster moving fraction was activated by addition of Mg^{2+}, it could be a contamination of fraction III. The slower moving fraction was probably due to the contamination of fraction I and the major activity was found between these two fractions.

Phospho-cellulose Column Chromatography

As fraction III was very unstable in alkaline pH even at 4°, the fraction was chromatographed on a phosphocellulose column (3×9 cm) equilibrated with 50 mM citrate buffer (pH 3.0) and eluted with a linear gradient with 300 ml of 50 mM citrate buffer (pH 3.0) and 300 ml of 50 mM citrate buffer containing 0.2M NaCl, pH 7.7. The enzymatic active fractions were collected. Three fractions I, II and III were chromatographed on Sephadex G-75 separately.

Sephadex G-75 Column Chromatography

The fraction I and II were eluted from the column at tube No. 76 and 79, respectively as a single enzymatic active peak. The fraction III eluted from phospho–cellulose column was eluted as two enzymatic active peaks. The elution position of the faster fraction coincided with that of fraction II, and the slower moving major peak was called fraction III hereafter (Fig. 4). The elution positions indicated that molecular weight of fractions I, II and III

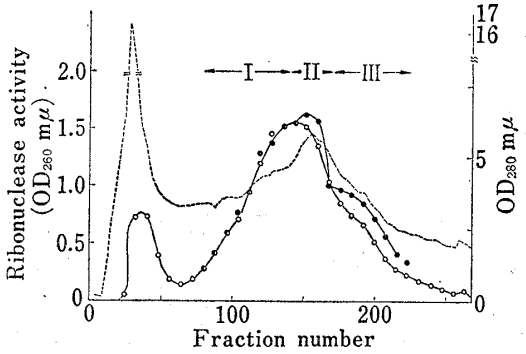

Fig. 1. IRC-50 Column Chromatography

IRC-50 column (4×20 cm) was equilibrated with 0.05M acetate buffer (pH 3.5). A gradient elution was performed with 2 liter of 0.05M acetate buffer (pH 3.5) and 2 liter of 0.1M acetate buffer (pH 6.5) containing 0.2M NaCl. each tube: 15 ml, About 23 g (3600 units) of protein was applied on the column, —○—: RNase activity in the absence of Mg^{2+}, —●—: RNase activity in the presence of Mg^{2+}, Fractions No. 80—140, 141—169 and 170—224 were designated as fraction I, II and III, respectively.

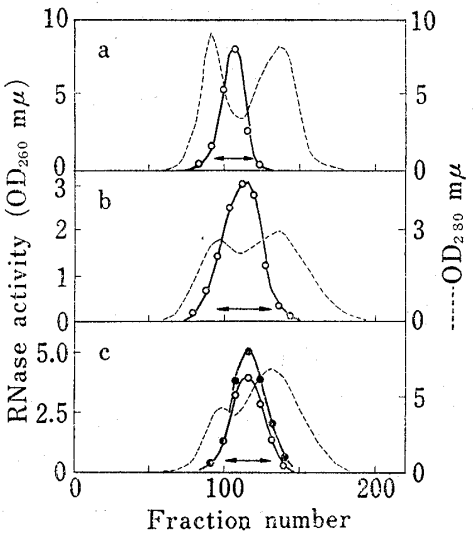

Fig. 2. Gel Filtration on Sephadex G-100

Sephadex G-100 (4×52 cm), buffer, 0.1M acetate buffer (pH 5.0), each tube: 5 ml, —○—: RNase activity in the absence of Mg^{2+} and —●—: RNase activity in the presence of Mg^{2+}

(a) Fractions having enzymatic activity obtained from the rechromatography of fraction I (Fig. 1) on IRC-50 (1380 units, 2.6 g protein) were applied on the column. (b) Fractions having enzymatic activity obtained from rechromatography of fraction II (Fig. 1) on IRC-50 column (610 units, 1.3 g) were applied on the column. (c) Fractions having enzymatic activity obtained from rechromatography of fraction III(Fig. 1) on IRC-50 column (670 units, 2.65 g protein) were applied on the column. The enzymatic activity was measured as described in the text.

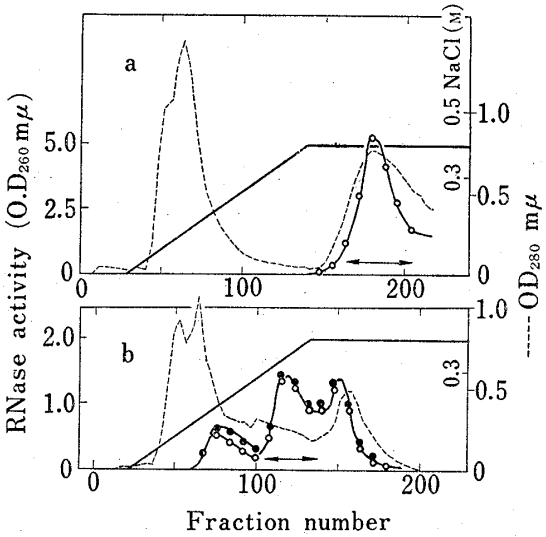

Fig. 3. DEAE-cellulose Column Chromatography

DEAE-cellulose (3×11.2 cm) was equilibrated with 5 mM phosphate buffer (pH 8.5). A linear gradient elution was performed with 300 ml of 5 mM phosphate buffer (pH 8.5) and 300 ml of 0.25M phosphate buffer (pH 4.0) containing 0.4M NaCl. each tube: 5 ml

(a) Enzymatic active fractions from Sephadex G-100 column of fraction I (Fig. 2,a) (1270 units, 610 mg protein) was applied. (b) Enzymatic active fractions from Sephadex G-100 column of fraction II (Fig. 2,b) (510 units, 420 mg protein) was applied. —●—: RNase activity in the presence of Mg^{2+}, and —○—: RNase activity in the absence of Mg^{2+}.

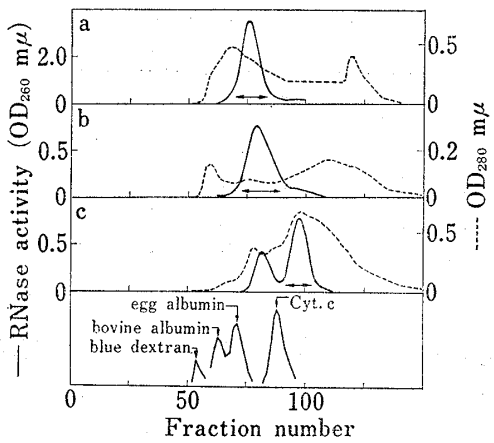

Fig. 4. Gel filtration on Sephadex G-75

Sephadex G-75 (3×94 cm) was equilibrated with 0.1M acetate buffer (pH 5.0). each tube: 5 ml

(a) Fractions having enzymatic activity obtained from DEAE-cellulose column chromatography of fraction I (Fig. 3,a) (800 units, 110 mg protein) were appli- ed on the column. (b) Fractions having enzymatic of fraction II (Fig. 3,b) (200 units, 35 mg protein) activity obtained from DEAE-cellulose column were applied on the column. (c) Fractions obtained from phospho-cellulose column chromatography, as described in the text (180 units, 150 mg protein) were applied on the column. The elution positions of the standard proteins, bovine serum albumin, egg albumin and horse heart cytochrome c were indicated at the bottom of the figure.

were approximately 24000, 18000 and 6000, respectively. These procedures, starting from the crude extract, resulted in 330, 260 and 40 fold purification for fractions I, II and III, respectively. The purification of RNases in each step is summarized in Table I. Three RNase fractions liberated less than 0.1 mμmole of *p*-nitrophenol from bis-*p*-nitrophenylphosphate or *p*-nitrophenylphosphate under assay conditions reported previously.[4] When desoxyribonucleic acid (DNA) was used as substrate, these enzymatic active fractions formed acid soluble nucleotide less than 0.1% of that formed when RNA was used as substrate. Therefore, these enzyme fractions were practically free from phosphomono- and di-esterase and DNase activities. However, trace amount of 3'-nucleotidase was found in these three fractions, since the longer incubation with RNA produces nucleosides in addition to nucleotides.

Table I. Purification of Ribonuclease from *Trichoderma koningii*

	Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)
1.	Crude extract	8240	138000	0.060	100
2.	0.30—0.85 saturation of ammonium sulfate precipitate	7110	45300	0.16	86
3.	IRC-50 column chromatography (I)				
	fraction I	2470	8130	0.30	30
	fraction II	1180	4910	0.24	14
	fraction III	1050	5980	0.18	13
4.	IRC-50 column chromatography				
	fraction I	1380	2640	0.52	17
	fraction II	610	1340	0.46	7.4
	fraction III	670	2650	0.25	8.1
5.	Sephadex G-100 column chromatography				
	fraction I	1270	610	2.1	15
	fraction II	510	420	1.2	6.2
	fraction III	500	770	0.66	6.1
6.	DEAE-cellulose column chromatography				
	fraction I	800	110	7.3	9.7
	fraction II	200	35	5.9	2.5
	Phospho-cellulose column chromatography				
	fraction III	180	150	1.2	2.2
7.	Sephadex G-75 column chromatography				
	fraction I	490	26	20	6.0
	fraction II	110	7.3	16	1.4
	fraction III	110	41	2.5	1.2

pH Optimum

When RNA was used as substrate, fractions I and II had the same pH optimum of 4.3 (Fig. 5), but that of fraction III was at pH 4.8 in the absence of Mg^{2+} and at pH 4.3 in the presence of $Mg.^{2+}$

Effect of Temperature on Enzymatic Activities of RNase Fractions I, II and III

Fig. 6 shows the effect of temperature on enzymatic activities under standard assay conditions. The activity maxima were found between 55—60° for each of three fractions.

Effect of Ionic Strength on Enzymatic Activity

The effect of ionic strength on the enzymatic activities of fractions I, II and III was studied using RNA as substrate at pH 5.0 (Fig. 7). The activities of fraction I and II were found to be maximum at ionic strength of 0.18. However, the activity of fraction III increased gradually with the increase of ionic strength, and the activity at ionic strength 1.0 was about 190% of that measured at ionic strength of 0.025.

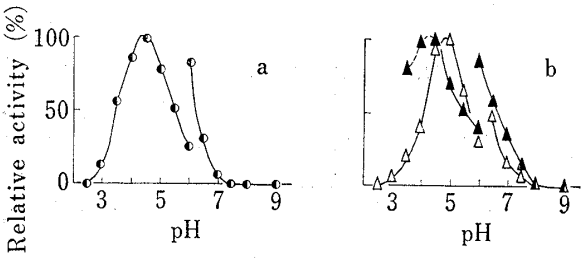

Fig. 5. Effect of pH on Enzymatic Activity

(a) Fraction I: —○—, fraction II: —●—
(b) Fraction III: —△—, in the absence of Mg^{2+} and —▲—, in the presence of Mg^{2+}. The assay conditions are the same as described in the text. The buffers used were citrate buffer (0.1M) for pH 2.5 and 3.0, acetate buffer (0.1M) for pH 3.5—6.0, imidazole-HCl buffer (0.1M) for pH 6.5 and 7.0 and Tris-HCl buffer (0.1M) for pH 7.5—9.0. Enzyme units used for assay were 0.19, 0.23 and 0.17 for fraction I, II and III, respectively.

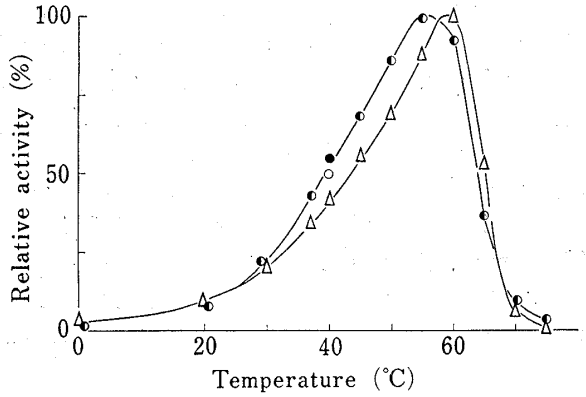

Fig. 6. Effect of Temperature on Activities of RNase Fractions I, II and III

The assay of activity was performed using the standard conditions (pH 5.0, see the text) at given temperature. Relative activity was expressed as per cent of maximum velocity (55° for fractions I and II, 60° for fraction III). Enzyme units used for assay were 0.19, 0.22 and 0.17 for fractions I, II and III, respectively. —○—: fraction I, —●—: fraction II and —△—: fraction III

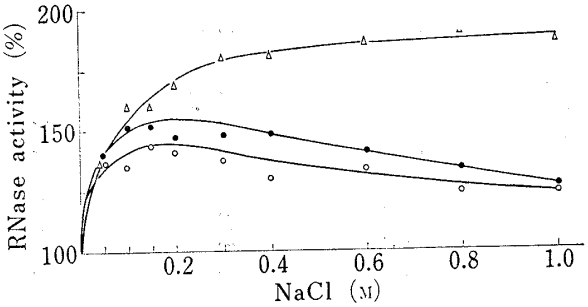

Fig. 7. Effect of Ionic Strength on Enzymatic Activity at pH 5.0

Assay conditions were as described in the text, except acetate buffer (0.025M) was used. The salt concentration was adjusted by addition of NaCl. Enzyme units used for assay were 0.19, 0.21 and 0.19 for fractions I, II and III, respectively. —○—: fraction I, —●—: fraction II and —△—: fraction III

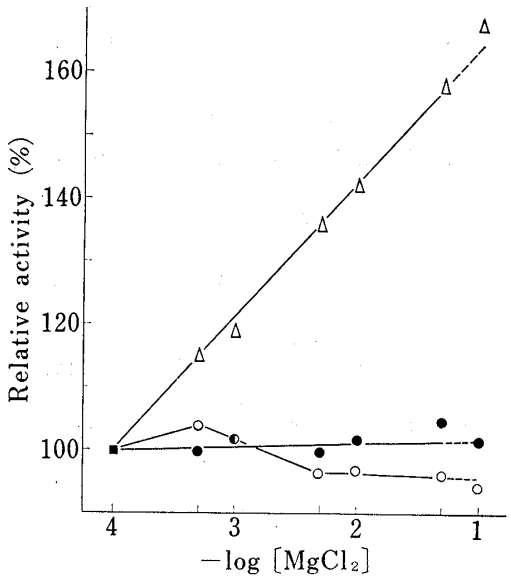

Fig. 8. Effect of Mg^{2+} concentration on Enzymatic Activities of Fractions I, II and III

The assay of activity was performed using standard conditions (see text) at given $MgCl_2$ concentrations. Relative activity was expressed as percent of activity at 10^{-4} M $MgCl_2$. Enzyme used for assay were 0.19, 0.23 and 0.20 units for fractions I, II and III, respectively. —○—: fraction I, —●—: fraction II, and —△—: fraction III

Effect of Divalent Cations and the Other Reagents

Hydrolyses of RNA by partially purified RNase fractions I and II were inhibited markedly by metal ions such as Cu^{2+}, Zn^{2+} and Hg^{2+} at the concentration of 10^{-3}M. However, hydrolysis of RNA by fraction III was inhibited by Cu^{2+} and Hg^{2+} very moderately at the same concentration, and was activated by Mg^{2+}, Ca^{2+} and Mn^{2+}. The activation by Mg^{2+} and Ca^{2+} at 10^{-2}M was about 150% of that in the absence of these metal ions (Table II). Activities of three RNase fractions from *Trichoderma koningii* were measured at variousconcentrations of Mg^{2+}. The activity of fraction III increased with the increase of Mg^{2+} up to 0.1M concentration (Fig. 8). The other divalent cations, ethylenediaminetetracetic acid (EDTA) and 2-mercaptoethanol did not affect the enzymatic activities of fractions I, II and III at the concentration of 10^{-3}M.

TABLE II. Effect of Divalent Cations and Other Reagents

Additions	Relative activity (%)								
	I			II			III		
	10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	12^{-4}	10^{-2}	10^{-3}	10^{-4}
$MgCl_2$		99			98		147	116	
$CaCl_2$		104			103		168	117	
$MnCl_2$		96			95			122	
$CoCl_2$		92			87			102	
$NiCl_2$		83			80			94	
$CuCl_2$		13			10			81	
$ZnCl_2$		31			31		84	115	
$CdCl_2$		89			86			107	
Ba(acetate)$_2$		104			100		105	112	
$HgCl_2$		40			40			77	
Pb(acetate)$_2$		105			96			105	
p-Chloromercuribenzoate			81			89			90
EDTA		93			103			100	
2-Mercaptoethanol	101			95			101		

The assay of activity was performed using the standard assay conditions (see the text) in the presence of divalent cations and other reagents. Relative activity was expressed as per cent of activity measured without additive. Enzyme used for assay were 0.19, 0.23 and 0.18 for fractions I, II and III, respectively.

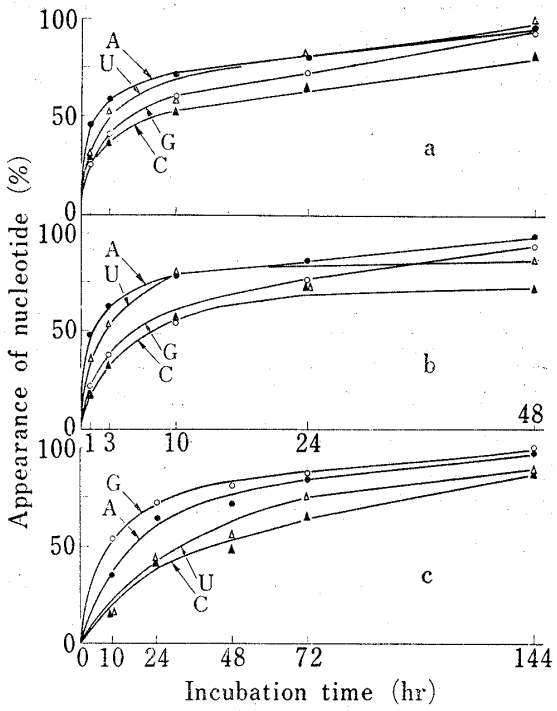

Fig. 9. Hydrolyses of RNA by RNase Fractions I, II and III

The experimental conditions were as described in the text. The amount of nucleotide appeared was compared with that formed by incubating with RNase from *Aspergillus saitoi*[4] which was known to hydrolyze RNA completely at the same concentration for 24 hr. (a) fraction I, (b) fraction II and (c) fraction III. —●—: 2′,3′-cyclic adenosine monophosphate (AMP)+3′—AMP+adenosine,[a] —○—: 2′,3′-cyclic guanosine monophosphate(GMP)+3′-GMP+guanosine,[a] —△—: 2′,3′-cyclic UMP+3′-UMP and —▲—: 2′,3′-cyclic cytidine monophosphate(CMP)+3′-CMP.

a) Nucleoside appeared was probably due to the contamination of 3′-nucleotidase in the enzyme preparations.

Substrate Specificity

After 1, 3, 10, 24 and 48 hours incubation, hydrolyzates of RNA by three enzyme fractions were chromatographed on Toyo Roshi No. 51 filter paper two dimensionally. Four common nucleotides and their cyclic nucleotides were observed in the hydrolyzates of RNA by three RNase fractions. The spots corresponding to four nucleotides and their cyclic nucleotides were cut and eluted with 0.1 N HCl for 18 hr at 30°. Then optical density of each eluate was measured. For fractions I and II, the spots corresponding to 2′,3′-cyclic nucleotides appeared at the initial stage of hydrolysis and they disappeared within 10 hr at the conditions described here. However, in

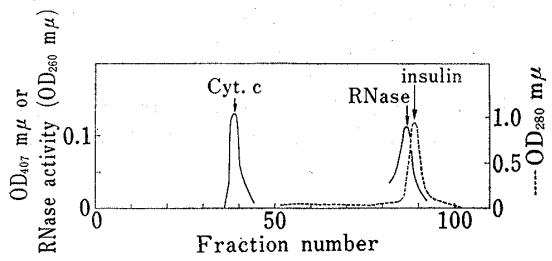

Fig. 10. Gel Filtration of Fraction III on Bio-Gel P-10

Bio-Gel P-10 (2.4×49.5 cm), buffer 0.1M acetate buffer, pH 3.5. Each tube, 2.5 ml. Fraction III loaded was 15.2 units (6.1 mg protein). Proteins used as standard were cytochrome c, 3.1 mg (MW 12750), insulin, 10.9 mg (MW 5773). Concentrations of proteins were measured by optical density at 280 nm for insulin and 407 nm for cytochrome c.

the case of fraction III, cyclic nucleotides remained even at 72 hr incubation. Especially 37% of cyclic uridine monophosphate (UMP) was hydrolyzed to 3'-UMP at 144 hr hydrolysis (not shown in this paper). As shown in Fig. 9, RNase fractions I and II liberated nucleotides in the order of adenylic acid, uridylic acid, guanylic acid and cytidylic acid and fraction III liberated in the order of guanylic acid, adenylic acid, uridylic acid and cytidylic acid.

Gel Filtration on Bio-Gel P-10

The elution pattern of fraction III enzyme from Sephadex G-75 indicated that the molecular weight of this enzyme is much smaller than cytochrome c. The molecular weight estimated by this figure was about 6000—7000. Gel filtration by Bio-Gel P-10 was performed to avoid the possibility that higher affinity of fraction III enzyme with Sephadex resin caused a retardation of this enzyme. The result was shown in Fig. 10. The RNase activity came out just before insulin.

Discussion

Fraction I and II which were non-specific in respect of bases released mononucleotides from RNA in the order of A>U>G>C and both enzymes have very similar specificity in spite of the difference in molecular weight. The order is quite similar to that of RNases from *Aspergillus saitoi*[4] and *Aspergillus oryzae* (RNase T_2).[6] However, the molecular weight of these enzymes are about 36000—38000 and different from those of fraction I and II. The fraction I enzyme has the same molecular weight as the base non-specific RNase isolated from *Rhizopus sp*,[7,8] but the latter enzyme liberated nucleotides from RNA in the order of A>G>C>U.

The result of Fig. 10 also indicated that RNase fraction III has a molecular weight of 6000. It is not possible to eliminate the possibility that some hydrodynamic properties of this enzyme were somewhat unusual and the molecular weight of the enzyme was underestimated. However, RNase which was eluted very close to insulin on gel filtration on Sephadex or Bio-Gel column is very rare so far as we know.

Fraction III liberated mononucleotides from RNA in the order of G>A>U>C. The order is similar to that of *Physarum polycephalum* RNases (RNase P_{p-2}, RNase P_{p-3}).[9] The molecular weight of *P. polycephalum* RNases were about 40000 for RNase P_{p-2} and 10000 for RNase P_{p-3}. From the fact that RNase fraction III has a molecular weight of about 6000 and is activated by Mg^{2+} and Ca^{2+} ions, RNase III could be a new type of RNase, since the most of RNases belonged to nucleotido-2'-transferase (cyclizing) are not accelerated by the addition of Mg^{2+} and Ca^{2+}. Since the content of fraction III in a commercial digestive "Meicelase P" was very low, fraction III was not purified completely yet. For further investigation of this enzyme, searches for the better cultivation conditions to yield higher RNase activity should be necessary.

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