米糠のアルカリ性リボヌクレアーゼの精製および性質

川　崎　良　文

Purification and Properties of an Alkaline Ribonuclease from Rice Bran

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INTRODUCTION

Ribonucleases (RNases) are found in a great number of higher plants. Those from pea leaves[1)2)], tobacco leaves[3)4)], rye grass[5)6)], wheat[7)], spinach[8)], soybean[9)], corn[10)11)], mung bean[12)13)14)], Japanese cycad[15)], and gingko nuts[16)] have been purified extensively and their modes of action on ribonucleic acid (RNA) were determined. It has been reported that these enzymes had pH optima in the acidic range and were able to cleave all the diester bonds in RNA to 2′,3′-cyclic mononucleotides. None of the plant RNases has so far been reported to have a strict base specificity as compared with *Aspergillus* RNase T_1[17)] and pancreatic RNase 1[18)].

It has previously been shown[19)] that at least three RNases were present in rice bran and that one of them, an acid RNase, had a pH optimum at 5.3 and hydrolyzed RNA to four 2′,3′-cyclic mononucleotides. This paper deals with the purification and some properties of an alkaline RNase from rice bran. The enzyme had a pH optium at 7.7 and was highly specific for guanylic acid residues in RNA.

EXPERIMENTALS

Materials. Freshly prepared rice bran was purchased from a farmer. Yeast RNA purchased from Nutritional Biochemicals Corp. was deprotenized by the method of Sevag et al.[20)] and used as a substrate for the assay of RNase. High-molecular-weight RNA was prepared by the method of Crestfield et al.[21)] from baker's yeast. After acid hydrolysis, the ratio of the constituent nucleotides in this RNA was found to be 27.6: 27.0: 19.8: 25.6 for adenylic acid: guanylic acid: cytidylic acid: uridylic acid. DEAE-cellulose (0.88 meq/g) was purchased from Brown Co. Sephadex G-75 (Medium) and CM-Sephadex C-50 (4.5 meq/g) were purchased from Pharmacia. Hydroxylapatite was prepared by the method of Tiselius et al.[22)].

Assay of RNase activity. The incubation mixture contained 0.20 ml of enzyme solution, 0.25 ml of 0.2 M Tris (tris (hydroxymethyl) aminomethane)-HCl buffer,

pH 7.7, 0.25 ml of 1.2% RNA solution, and 0.30 ml of water. After 15 minutes at 37°C, 0.20 ml of 25% perchloric acid containing 0.75% uranyl acetate was added to stop the reaction. The mixture was allowed to stand for 30 minutes at room temperature and the precipitate was removed by centrifugation for 10 minutes at 2,500 rpm. To 0.20 ml of the supernatant 4.80 ml of water was added, and the absorbancy at 260 mμ (A_{260}) of the solution was measured in a Hitachi EPU-2A spectrophotometer. This assay was linear up to A_{260} values of 0.5. One unit of RNase activity is defined as the amount of enzyme which causes a change in A_{260} of 1.0 under the above conditions. The specific activity is defined as the units per mg of protein. Protein concentration was determined by spectrophotometry based on the assumption that 1 mg per ml solution has an A_{280} of 1.0 except that the biuret method[23] was used for the crude extract because of the presence of non-protein impurities.

Purification of the enzyme.

Step 1. All steps in enzyme fractionation were carried out at 4°C. Sixteen hundred grams of rice bran was homogenized for 5 minutes in a 3-liter Waring blender in four equal batches with a total volume of 8 liters of 0.2 M NaCl. The homogenate was squeezed through double layers of cheesecloth and the filtrate was centrifuged at 13,000×g for 10 minutes. The layer of material floating on the surface of the supernatant solution was skimmed off and the solution obtained was designated as the crude extract.

Step 2. The crude extract was brought to 0.4 saturation by the addition of 287 grams of solid ammonium sulfate per liter of the solution. After standing overnight, the solution was centrifuged at 15,000×g for 10 minutes, and the turbid supernatant was clarified with the aid of Celite No. 535. The clear brown filtrate was brought to 0.6 saturation by the addition of 124.5 grams of ammonium sulfate per liter of the solution. The solution was left overnight and then centrifuged at 13,000×g for 10 minutes. The precipitate was suspended in about 100 ml of water and dialyzed for 3 days against several changes of 0.005 M sodium phosphate buffer, pH 7.1. The insoluble material which formed during dialysis was removed by centrifugation and discarded. The supernatant was stored at −20°C without appreciable loss in the activity for several months.

Step 3. The ammonium sulfate fraction was loaded on a DEAE-cellulose column (3.8×50 cm) previously equilibrated with 0.005 M sodium phosphate buffer, pH 7.1. A linear gradient was carried out from 1.5 liters of 0.005 M sodium phosphate buffer, pH 7.1, to 1.5 liters of 0.4 M NaCl in 0.1 M sodium phosphate buffer, pH 5.0. As is evident from Fig. 1, RNA depolymerizing activity was separated into four peaks. The first peak was shown to have phosphodiesterase activity, whereas the others not. So the peaks, lettered A, B, and C, were named rice bran RNase A, RNase B, and RNase C, respectively. The RNase A

fraction (tubes 39 to 48) was made 0.9 saturation with ammonium sulfate. The precipitate was collected by centrifugation and filled up to 20 ml with water.

Step 4. The DEAE-cellulose fraction was allowed to pass through a Sephadex G-75 column (3.8×55 cm) equilibrated with 0.1 M NaCl in 0.01 M ammonium acetate, pH 6.8 (Fig. 2). Two batches, correspond to 3.2 kilograms of starting material, from the gel filtration were combined and concentrated in Visking cellophane tube against solid ammonium sulfate. The collected precipitate was dissolved in a minimum volume of water and dialyzed, with stirring, for several hours against 1 liter of 0.1 M Tris-acetate buffer, pH 6.0.

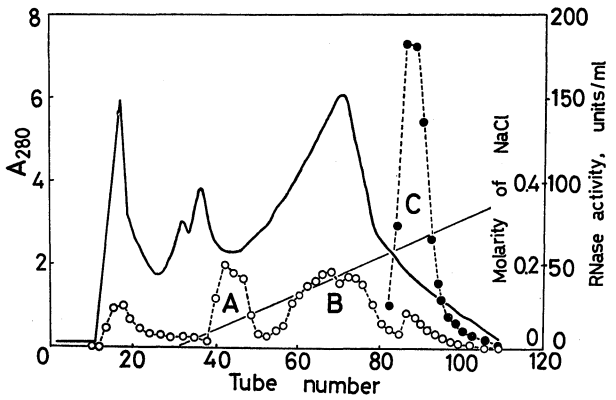

Fig. 1. Chromatography of the ammonium sulfate fraction on DEAE-cellulose column (3.8×50 cm). Linear gradient was carried out from 1.5 l of 0.005 M sodium phosphate, pH 7.1, to 1.5 l of 0.4 M NaCl in 0.1 M sodium phosphate, pH 5.0. Flow rate, 60 ml/hr; 25.7 ml/tube. ——, absorbancy at 280 mμ (left-hand scale) and molarity of NaCl (right-hand inner scale); ···○···, RNase activity at pH 7.7; ···●···, RNase activity at pH 5.3. Peaks A, B, and C were named rice bran RNase A, RNase B, and RNase C, respectively.

Step 5. The Sephadex fraction was loaded on a CM-Sephadex C-50 column (1.8×45 cm) equilibrated with 0.1 M Tris-acetate buffer, pH 6.0. A linear gradient was carried out from 500 ml of 0.1 M Tris-acetate buffer, pH 6.0, to 500 ml of 0.2 M Tris-acetate buffer, pH 8.1. As shown in Fig. 3, RNase A was imper-

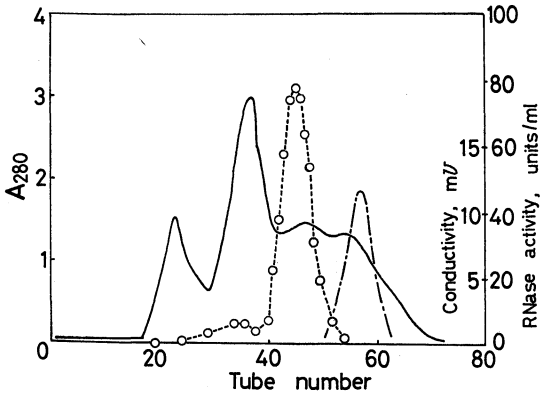

Fig. 2. Gel filtration of the DEAE-cellulose fraction through Sephadex G-75 column (3.8×55 cm). Eluting solution, 0.1 M MaCl in 0.01 M ammonium acetate, pH 6.8. Flow rate, 30 ml/hr; 10 ml/tube. ——, absorbancy at 280 mμ; ···○···, RNase activity; —·—·—, conductivity which indicates the peak of ammonium sulfate in the sample.

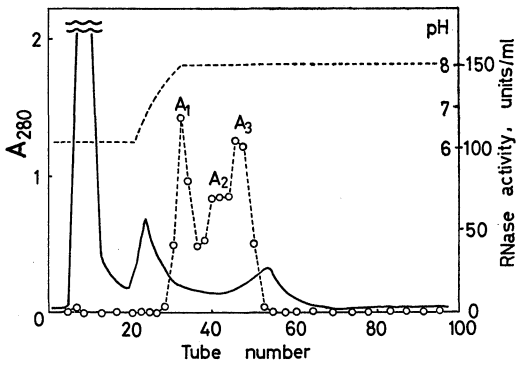

Fig. 3. Chromatography of the Sephadex fraction on CM-Sephadex C-50 column (1.8×45 cm). Linear gradient was carried out from 500 ml of 0.1 M Tris-acetate buffer, pH 6.0, to 500 ml of 0.2 M Tris-acetate buffer, pH 8.1. Flow rate, 20 ml/hr; 10 ml/tube. ——, absorbancy at 280 mμ; ···○···, RNase activity; ·····, effluent pH.

fectly separated into three components (A_1, A_2, and A_3).

Step 6. The CM-Sephadex fractions, Component A_1 and Component A_2 plus A_3, were loaded on hydroxylapatite columns (1.0×16 cm) equilibrated with 0.01 M sodium phosphate buffer, pH 6.8. A linear gradient was carried out from 100 ml of 0.01 M sodium phosphate buffer, pH 6.8, to 100 ml of 0.1 M sodium phosphate buffer, pH 6.8. Component A_1 was eluted in one peak and Component A_2 plus A_3 was eluted in two peaks in activity as shown in Fig. 4. A summary of the purification of rice bran RNase A is given in Table 1. The enzyme was purified 280- to 460-fold. Since different RNase activities were found in the crude extract, the total activity of this extract represents an apparent amount.

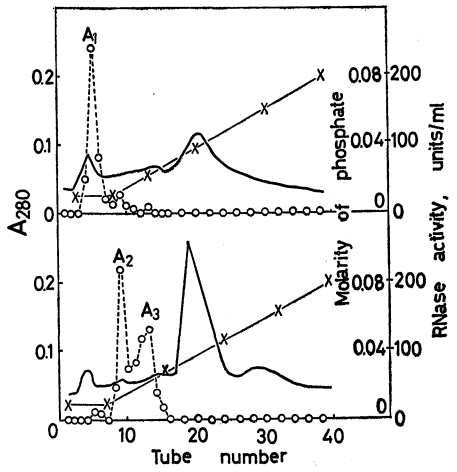

Fig. 4. Chromatography of Componet A_1 (top) and Component A_2 plus A_3 (bottom)on hydroxylapatite column (1.0×16 cm). Linear gradient was carried out from 100 ml of 0.01 M sodium phosphate buffer, pH 6.8, to 100 ml of 0.1 M sodium phosphate buffer, pH 6.8. Flow rate, 5 ml/hr; 5.3 ml/tube. ——, absorbancy at 280 mμ; ···○···, RNase activity; —×—, molarity of phosphate.

RESULTS

Properties. The hydroxylapatite fractions, Comonent A_1 and Component A_2 plus A_3, were used throughout the following investigations unless otherwise indicated.

PH optimum - The pH optima for both components were observed at 7.7 as shown in Fig. 5.

Temperature optimum - The temperature optima for both components were found at about 45°C as shown in Fig. 6.

Effect of substances on activity - The effect of various substances on activity was studied. As is evident from Table 2, the two components were found to show similar behaviors towards various substances. They were not activated by any

Table 1. Summary of purification procedure

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity	Yield (%)
1. Crude extract	6,560	37,800	132,000	3.5	100
2. Ammonium sulfate (0.4-0.6 satn.)	135	7,530	57,500	7.6	43
3. DEAE-cellulose	20	748	7,130	9.5	5.4
4. Sephadex G-75	4	91.3	5,130	56	3.9
5. CM-Sephadex Component A_1		5.3	1,790	340	3.3
5. CM-Sephadex Component A_2 plus A_3		7.0	2,540	360	
6. Hydroxylapatite Component A_1		0.44	630	1,400	1.2
6. Hydroxylapatite Component A_2		0.29	470	1,600	
6. Hydroxylapatite Component A_3		0.58	570	980	

Sixteen hundred grams of rice bran was used as starting material.

Table 2. Effect of substances on the activities of Component A_1 and Component A_2 plus A_3

Substance (10^{-3}M)	Relative activity (%)		Substance (10^{-3} M)	Relative activity (%)	
	Component A_1	Component A_2 plus A_3		Component A_1	Component A_2 plus A_3
None	100	100	$ZnCl_2$	32	35
EDTA*	98	97	$SnCl_2$	59	44
KCl	86	89	$AgNO_3$	25	35
$BaCl_2$	97	91	$NaHSO_3$	88	94
$CaCl_2$	83	94	NaF	97	95
$MgCl_2$	81	94	NaCN	100	96
$Mn(CH_3COO)_2$	99	93	Sodium phosphate	104	98
$CoCl_2$	62	68	Thiourea	102	91
$NiSO_4$	57	67	Mercaptoethanol	105	81
$FeSO_4$	90	107	Cysteine	91	102
$FeCl_3$	97	93	CH_2ICOOH	91	107
$HgCl_2$	47	57	PCMB**	93	96
$CuSO_4$	46	58			

* Ethylenediaminetetraacetate　** p-Chloromercuribenzoate

of the substances tested, and were inhibited by $HgCl_2$, $CuSO_4$, $ZnCl_2$, $SnCl_2$, and $AgNO_3$.

Heat stability - The two components in 0.01 M acetate buffer, pH 5.5, containing 0.1 M NaCl were shown to retain 95 percent of their activities in a boiling water for 5 minutes.

Estimation of molecular weight - The approximate molecular weight of rice bran RNase A was determined by the gel filtration method as in the previous paper[19] and was found to be 11,000. The ratio of elution volume to void volume of the enzyme was 2.52.

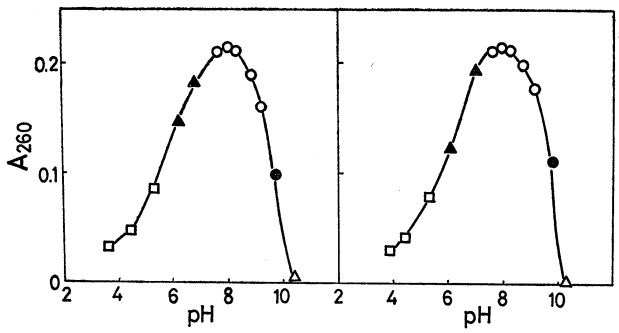

Fig. 5. PH optima of Component A_1 (left) and Component A_2 plus A_3 (right). Buffers used: —□—, acetate; —▲—, phosphate; —○—, Tris-HCl; —●—, glycine-NaOH; —△—, carbonate.

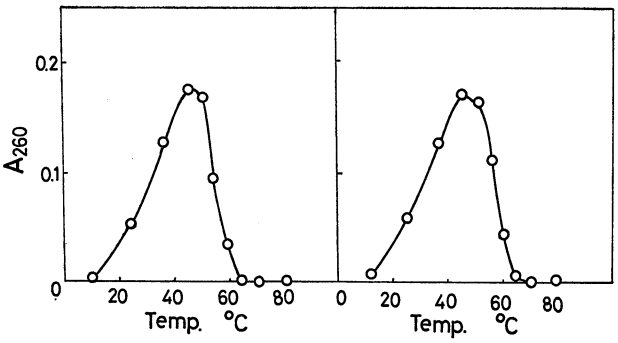

Fig. 6. Effect of temperature on the activity of Component A_1 (left) and Component A_2 plus A_3 (right).

Contaminating enzymes - For specificity studies RNase preparation should be free of contaminating enzymes. The hydroxylapatite fractions were shown to be free of deoxyribonuclease, phosphodiesterase, and phosphomonoesterase activities incubated for 18 hours at 37°C. The deoxyribonuclease activity was measured by the method of Mukai[24]. The phosphodiesterase and phosphomonoesterase activities were measured essentially the

same method of Laskowski et al.[25] except that buffer solutions were replaced by the acetate buffers of pH 3.5 and 5.3, respectively.

Base specificity.

Base specificity at 3′-termini - The reaction mixtures (total volumes of 1.0 ml) each containing 0.25 ml of high-molecular-weight RNA solution (20 mg per ml), 0.25 ml of 0.2 M Tris-HCl buffer, pH 7.7, and about 20 units or 100 units of each enzyme component were incubated for 24 hours at 37°C with a few drops of toluene. An identical incubation without enzyme was used as a control. The mixtures were then kept 2 hours in 0.1 N HCl at 37°C[17] to split any 2′, 3′-cyclic phosphate esters if present. After neutralization, the mixtures were adjusted to pH 8 with 2 M Tris. To 0.6 ml aliquots about 50 μg of bacterial alkaline phosphatase (Nutritional Biochemicals Corp.) was added to split the terminal phosphate groups of the digests, and the mixtures were kept for 4 hours at 37°C. Alkaline hydrolysis was then carried out in 1 N KOH for 18 hours at 37°C. The hydrolyzates were adjusted to pH 3 with perchloric acid and desalted by charcoal columns[26]. About 8 A_{260} units of each sample was applied to Toyo Roshi No. 51A paper and was subjected to two-dimensional paper chromatography by the descending technique. The solvent systems were 95% ethanol-1 M ammonium acetate, pH 7.5, (75 : 30)[27] in the first dimension and isobutyric acid-0.5 M ammonium hydroxide, pH 3.6,[28] in the second.

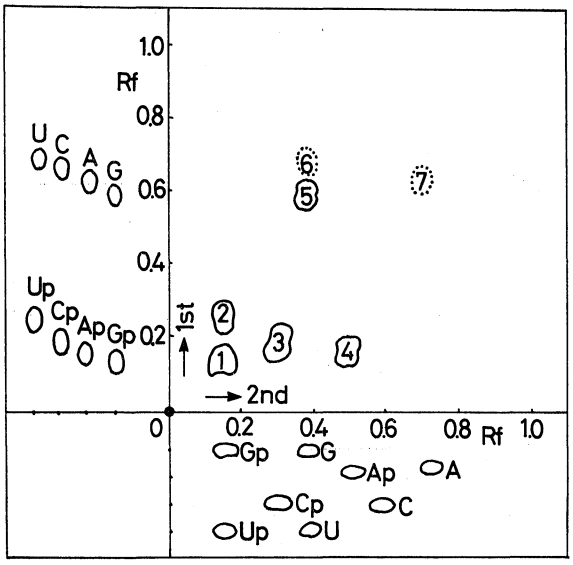

Fig. 7. Paper chromatography of nucleosides and nucleotides obtained by rice bran RNase A, alkaline phosphatase, and alkaline digestions. Authentic compounds were used as markers. First dimension, 95% ethanol-1 M ammonium acetate, pH 7.5, (75 : 30). Second dimension, isobutyric acid-0.5 M ammonium hydroxide, pH 3.6. Abbreviations : A, G, C, and U indicate adenosine, guanosine, cytidine, and uridine, respectively. The letter p written to the right of the symbol for a nucleoside indicates 2′-and/or 3′-phosphate. Spots 1 to 7 were identified as Gp, Up, Cp, Ap, G, U, and A, respectively.

A typical paper chromatogram is shown in Fig. 7. The spots were viewed under ultraviolet light and were eluted with 5 ml of 0.01 N HCl for 2 days at room temperature. The amount of the compound eluted from each spot was determined spectrophotometrically. The results are given in Table 3. The compounds were identified by a comparison of their Rf values with those of authentic compounds and their A_{250}/A_{260}, A_{280}/A_{260}, and A_{290}/A_{260} ratios at pH 2 and 7[29]. When the base frequency was calculafed from Table 3, more than 87 percent of the guanine which is originally present in RNA

Table 3. Analysis of base distribution in the digestion fragments

Digest	A_{260} units*×1000							
	3′-terminus				the rest (5′-terminus+internal)			
	G	A	C	U	Gp	Ap	Cp	Up
Control	0	0	0	0	2225	2620	845	1825
Component A_1, 20 units**	2010	45	0	50	295	2820	915	1960
Component A_1, 100 units	2500	70	0	5	90	3180	1040	2135
Component A_2, 16 units	2190	50	0	45	165	2955	905	1995
Component A_3, 20 units	2085	65	0	60	195	2680	880	1845

* One A_{260} unit is the amount of compound which gives an A_{260} of 1 in 1 ml of solution in a 1-cm light path.
** Units of enzyme per 5 mg of yeast RNA.

was responsible for guanosine at the 3′-termini, whereas less than 3.2 percent of the other bases was responsible for their corresponding nucleosides. The standard deviation of this analysis was ±1.7 percent. Accordingly, the very low values for the 3′-terminal nucleosides may fall within a limit of experimental error. From these data, it may be concluded that all the three components of rice bran RNase A showed the same specificity for guanylic acid linkages in RNA.

Identification of the mononucleotides in enzymatic digests of RNA - From the preceding data, it is not yet clear whether the enzyme is a 3′-(or 2′-)monoester former or a 5′-monoester former. To answer this question the following experiment was made. The reaction mixture (total volume of 2.25 ml) containing 25 mg of high-molecular-weight RNA, 0.25 ml of 0.2 M Tris-HCl buffer, pH 7.7, and 200 units of the enzyme (a mixture of equal amounts of Components A_1, A_2, and A_3) was incubated for 24 hours at 37°C. The mixture was then extracted three times with an equal volume of phenol. The last traces of phenol were removed from the aqueous solution by extraction with ether, and the ether was evaporated. A 1.0 ml aliquot was mixed with 0.4 grams of urea and 7 ml of 7 M urea, and the solution was loaded on a DEAE-cellulose column (1.0×37 cm) equilibrated with 0.02 M sodium acetate in 7 M urea, pH 7.5. A linear gradient was carried out from 250 ml of 0.02 M sodi-

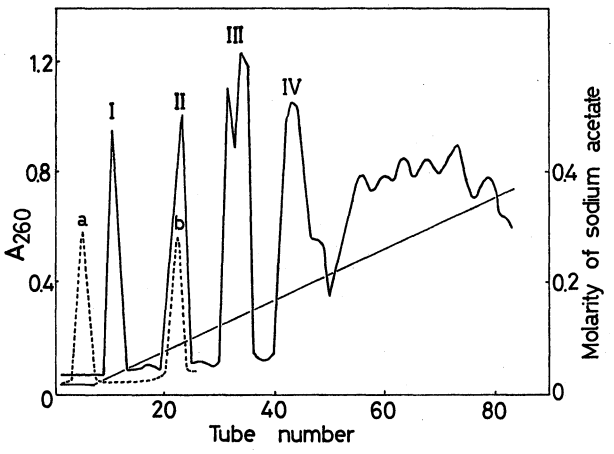

Fig. 8. Chromatography of rice bran RNase A digests of RNA on DEAE-cellulose column (1.0×37 cm). Linear gradient was carried out from 250 ml of 0.02 M sodium acetate in 7 M urea, pH 7.5, to 250 ml of 0.4 M sodium acetate in 7 M urea, pH 7.5. Flow rate, 9.5 ml/hr; 6 ml/tube. ——, absorbancy at 260 mμ (left-hand scale) and molarity of sodium acetate (right-hand scale); ……, markers of adenosine (a) and adenylic acid plus guanylic acid (b).

um acetate in 7 M urea, pH 7.5, to 250 ml of 0.4 M sodium acetate in 7 M urea, pH 7.5. The column chromatogram is shown in Fig. 8. The digests were fractionated on this column according to the degree of net charge as described by Tomlinson and Tener[30].

Peak I, eluted between the peaks of authentic nucleoside and nucleotide, was not adsorbed on a DEAE-cellulose column which was used for the desalting of mono- and oligonucleotides by Rushizky and Sober[31]. Hence, it was supposed to be cyclic mononucleotides. It was then treated with 0.1 N HCl for 2 hours at 37°C and was desalted by a charcoal column[26]. The eluate was concentrated in a vacuum to dryness. The sample was separated by two-dimensional paper chromatography. The solvent systems were isobutyric acid - 0.5 M ammonium hydroxide, pH 3.6, in the first dimension (descending) and saturated ammonium sulfate - 1 M sodium acetate - isopropanol (80: 20: 2)[32] in the second (ascending). Only two spots corresponding to 2′- and 3′-guanylic acids were obtained from the acid-treated peak I. Thus, peak I was considered to be originally the 2′, 3′-cyclic guanylic acid.

Peak II, eluted at the peak of authentic mononucleotide, was desalted by a DEAE-cellulose column[31] and concentrated. Aliquot of the sample was applied, directly or after the acid treatment, to the filter paper. One-dimensional descending paper chromatography was accomplished with 40 grams of ammonium sulfate in 100 ml of 0.1 M sodium phosphate, pH 7.0,[33] as a solvent. As is evident from Fig. 9 (a), three spots were obtained. One of them (4) was not affected its Rf value by the acid treatment, while the others were affected. To further investigate the mononucleotide-containing sample, two-dimensional descending paper chromatography was carried out. The solvent systems were isopropanol - water (70: 30), ammonia in vapor phase,[34] in the first dimension and the same solvent as in Fig. 9 (a) in the second. The paper chromatogram is shown in Fig. 9 (b). Only one spot corresponding to 3′-guanylic acid was detected as mononucleotides. Although the other spots were not identified, they were assumed to

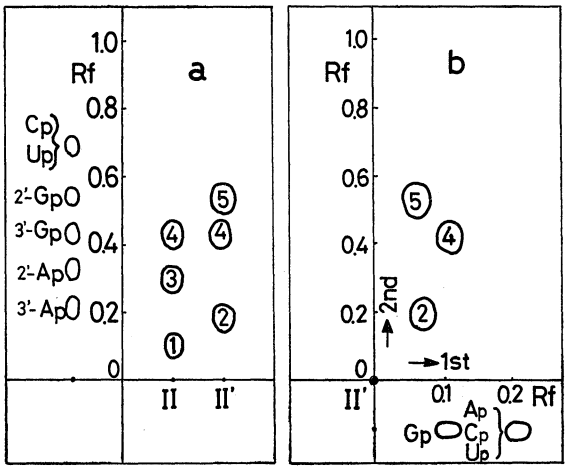

Fig. 9. Paper chromatography of peak II. Sample: II, intact; II′, acid-treated. Authentic nucleotides were used as markers. Solvents: (a), 40 g of ammonium sulfate in 100 ml of 0.1 M sodium phosphate, pH 7.0; (b), first dimension, isopropanol-water (70: 30), ammonia in vapor phase; second dimension, the same as (a). Spots: 1, (ApGp!); 2, (ApGp); 3, (CpGp! and/or UpGp!); 4, 3′-Gp; 5, (CpGp and/or UpGp). Parentheses indicate hypothetical identifications. The symbol ! indicates 2′, 3′-cyclic phosphate.

be ApGp! and CpGp! and/or UpGp! on the basis of the Rf values reported by Rushizky and Sober[33] and of the base specificity of this enzyme.

It is evident from these data that the products obtained by the action of the enzyme on RNA are 3′-isomers. In this experiment, the amounts of 2′, 3′-cyclic guanylic acid and 3′-guanylic acid found in the digests were 11% and 3% of the total guanylic acids in RNA, respectively.

DISCUSSION

The ammonium sulfate fraction of rice bran was separated into three peaks of RNase activity by chromatography on DEAE-cellulose. An alkaline RNase, named rice bran RNase A, was first eluted. This enzyme was then passed through Sephadex G-75 and was further separated into three components (A_1, A_2, and A_3) by chromatography on CM-Sephadex and on hydroxylapatite. The chromatograms of the Sephadex fraction on CM-Sephadex were found to vary with the eluting buffer systems used[35]. When phosphate buffer or ammonium acetate buffer was used instead of the Tris-acetate buffer, the enzyme was poorly separated from impurities and was not separated into any component. It is not clear, however, whether the fractionation behavior is due to the chromatographic process or to nature of the enzyme. All the three components of the enzyme were essentially identical in their properties, whereas they were chromatographycally different from one another.

Most of the plant RNases having pH optima in the acidic range are able to cleave all the diester bonds in RNA to 2′, 3′-cyclic mononucleotides. RNases having pH optima in the alkaline range have been described from several sources; the RNases from alfalfa and berseem had a pH optimum at 7.5 to 7.6[36], the minor fraction of the RNases from rye seedlings had a pH optimum at 8.0[37]. However, these enzymes have not yet been characterized their base specificities.

The alkaline RNase from rice bran has a pH optimum at 7.7. With respect to the base specificity of this enzyme, large amounts of guanosine and minute amounts of the other nucleosides were observed at the 3′-terminal position of the enzymatic digests of RNA. Moreover, the mononucleotides formed by the action of the enzyme on RNA were only 2′, 3′-cyclic and 3′-guanylic acids. Accordingly, it may be concluded that the enzyme specifically hydrolyzes phosphodiester bonds between 3′-guanylic acid and other nucleotides in RNA via a 2′, 3′-cyclic guanylic intermediate. Its base specificity appears to be practically the same as RNase T_1. Another point of interet is that both rice bran RNase A and RNase T_1[38] have a molecular weight of about 11,000.

SUMMARY

An alkaline ribonuclease was purified from rice bran by fractionation with ammonium sulfate, chromatograbhy on DEAE-cellulese, and gel filtration through Sephadex G-75. The enzyme was further purified and separated into three components, which were essentially identical in their properties, by chromatography on CM-Sephadex C-50 and on hydroxylapatite.

The enzyme has a pH optimum at 7.7 and a temperature optimum at about 45°C, and is heat stable. A molecular weight of about 11,000 is estimated for the enzyme by gel filtration. The purified enzyme is free of deoxyribonuclease, phosphodiesterase, and phosphomonoesterase activities.

The base specificity of the enzyme was elucidated by determining the base frequency at 3′-terminal position of the enzymatic digests of ribonucleic acid and by identifing the monocleotides in the digests. The enzyme specifically hydrolyzes phosphodiester bonds between 3′-guanylic acid and other nucleotides in ribonucleic acid via a 2′, 3′-cyclic guanylic intermediate.

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