原　著

花粉の生化学的研究 XXV
アカマツ花粉リボヌクレアーゼの多形体

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Biochemical studies on pollen　XXV

Multiple forms of ribonuclease in the pollen of *Pinus densiflora* Sieb. et Zucc.

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Introduction

In the course of biochemical studies on the pollen of *Pinus densiflora* Sieb. et Zucc. (pine pollen), we found that the activities of adenylate cyclase,[1] cyclic nucleotide phosphodiesterase[2] and acid phosphatase[3] increased during germination, and that the syntheses of invertase, glucose-6-phosphate dehydrogenase and acid proteinase in germinating pollen partly depended on endogenous preformed messenger RNA.[4] Furthermore, it was observed that a large quantity of RNA is found particularly in the tip of the germinated pollen tube,[5] and that the amount of cyclic AMP,[6] cyclic GMP[6] and acid soluble nucleotide,[7] such as ATP, UTP, GTP and uridine diphosphate glucose, increased during germination.

The RNA synthesis in germinating pollen has been reported by many investigators using the pollen of several species, tobacco[8~12], *Tradescantia paldosa*,[13~15] *Petunia hybrida*,[16] *Lilium longiflorum*,[17,18] *Amaryllis vittata*[19] and *Malus domestica*.[20] Moreover, the existence of preformed messenger RNA and its utilization at an early stage of germination has been observed using pollens of several species, *Impatiens balsamina*,[21] *Amaryllis vittata*,[19] *Tradescantia paldosa*[21,22] *and Arachis hypogaea*,[23] Recently, Tupy *et al.* reported ribosomal RNA synthesis[24] and transfer RNA synthesis[25] in germinating pollen of *Nicotiana tabacum.*

These phenomena may be concerned with the drastic and complex metabolism of nucleotides and RNAs during the germination of pollen.

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Knox *et al*.[26,27] detected, using cytochemical methods, the activities of acid phosphatase, ribonuclease (RNase) and esterase in the wall of pollen of many angiosperm species and one gymnosperm, *Pinus banksiana*. They demonstrated that these enzymes were associated with the intine, the inner cellulosic part of the cell wall, and that the intine enzymes are very readily leachable. Singh *et al*.[28] detected also cytochemically RNase activity in *Pinus roxburghii* pollen. In the case of Japanese cycad pollen,[29] RNase was released from the cell-surface with 0.5M sodium chloride solution, and the partially purified RNase degraded RNA giving rise to 2′, 3′-cyclic mononucleotide and 3′-mononucleotide.

Although cytochemical localization of RNase in the wall of the pollen grain is reported and the activity of RNase is detected in the extracts from pollen of Japanese cycad[29] and rape,[30,31] little or no information is obtained about the properties and multiple forms of RNase of matured or germinated pollen.

The present investigation describes the partial purification and some properties of multiple forms of RNase A which is released from the cell-surface of pine pollen with 0.5M sodium chloride solution, and of RNase B which is obtained from the residual fraction of the 0.5M sodium chloride extraction by suspension in 0.1M phosphate buffer, pH5.5, and disruption with a French Press. This is the first report of multiple forms of RNase of pollen.

The changes in RNase activity during the germination of pine pollen will be published elsewhere.

Materials and Methods

Materials. pollen of *Pinus densiflora* Sieb. et zucc. was collected from the anther just before dehiscence and stored at −20°C as described previously.[32] The following materials were purchased from the sources indicated in parentheses: DEAE-cellulose (Brown, Seikagaku Kôgyô), Sephadex G-75, Sephadex G-100, Sephadex G-200 (Pharmacia Fine Chemicals), standard proteins (Schwarz-Mann), yeast RNA (Boehringer), herring sperm DNA (Wako Chemical Industries), poly(A), poly(C) and poly(U) (Yamasa Shoyu). Other reagents used were of analytical grade. Bull semen 5′-nucleotidase was a generous supply of Yamasa Shoyu Co.

Assay for ribonuclease. The reaction mixture containing 75μl of 0.1M phospate buffer, pH5.5, 75μl of 0.5% yeast RNA in the same buffer, 50μl of water and 50μl of enzyme solution, was reacted at 37°C for 1 hr and terminated by adding 50μl of 25% perchloric acid containing 0.75% uranyl acetate. After centrifugation at 10,000×g for 10min, 2 ml of water was added to 50μl of the supernatant obtained, and then the absorbance was determined at 260nm against a blank incubated without enzyme. One unit of RNase activity was defined as the amount of enzyme that causes an increase in absorbance of 1.00 at 260nm under the above conditions.

Assay for protein. The amount of protein was determined by the method of Bradford,[33] using bovine serum albumin as a standard.

Estimation of molecular weight. The molecular weight of RNases was estimated by gel filtration according to the method of Andrews.[34] A Sephadex G-100 column (0.6×30cm) equilibrated beforehand with 0.05M Tris-HCl buffer, pH7.2, containing 0.1M NaCl, was used. Samples in 0.5ml of the same buffer

were applied to the column. The flow rate was 40 drops (about 1.6ml) per 1 hr and 5 drops portion was collected by a Gilson micro fraction collector.

Test of endo-or exonuclease activity. Test of endo- or exonuclease activity was performed according to the method of Birnboim [35] The reaction mixture containing 550μl of 0.25% poly(U) or poly(C) in 0.1M phosphate buffer, pH5.5, and 300μl of enzyme solution, was incubated at 37°C for the times indicated in Fig. 10. After the reaction, to a 10μl sample was added 100μl of 50mM Tris-HCl buffer, pH7.2, containing 0.1M NaCl, and the sample was applied to a Sephadex G-75 column (0.3×60cm) equilibrated with the same buffer and eluted with the same buffer. The flow rate was 200μl per min and absorption of the elutes was measured continuously at 260nm by UV absorbance (Tôyô model 400G).

Analysis for the enzyme-digestion products. The reaction mixture containing 50μl of 0.2M acetate buffer, pH5.5, 100μl of 1% yeast RNA and 100μl of enzyme solution, was incubated at 37°C for 35 hr and terminated by heating in boiling water for 3 min. After centrifugation at 1,500×g for 20 min, the supernatant was used for the determination of digestion products. Some parts of the supernatant obtained were treated with bull semen 5′-nucleotidase as follows. The reaction mixture contained 100μl of 0.5M Tris-HCl buffer, pH9.0, 100μl of bull semen 5′-nucleotidase solution, 25μl of 0.4M $MgCl_2$ and 125μl of the supernatant. The reaction was carried out at 45°C for 3 hr and terminated by heating in boiling water for 3 min. After filtration through a membrane filter, the filtrate was used for the determination of digestion products. The digestion products were determined by high performance liquid chromatography (HPLC, Hitachi HPLC, model T-635) under the following conditions. Hitachi custom resin # 2632 was packed into a column (2.1×500mm) and equilibrated with 0.01M NaCl in 5×10^{-3}N HCl. Sample was applied to the column and eluted with a linear gradient of 0.01M NaCl in 5×10^{-3}N HCl to 0.03M NaCl in 1.5×10^{-2}N HCl (total 10ml). The flow rate was 0.8ml per min. Temperature of the column was 80°C.

Results

Purification of RNase A from pine pollen

One hundred grams of ungerminated pollen were suspended in 10 volumes of 0.5M sodium chloride solution and shaken at room temperature for 45 min. After the treatment with 0.5M sodium chloride solution, it was observed that the ungerminated pollen grains were not disrupted. The resulting residue was removed by filtration through a filter paper (Tôyô, No.2). The residue obtained was washed with 100ml of 0.5M sodium chloride solution. The filtrate and the washings were combined and centrifuged at 17,000×g for 15min. The supernatant was brought to 0.7 saturation of ammonium sulfate. After standing overnight, the precipitate was collected by centrifugation at 17,000×g for 20 min, and dissolved in 0.01M phosphate buffer, pH5.5, and dialyzed overnight against the same buffer. After centrifugation of the dialyzate at 26,000×g for 10 min, the supernatant was applied to a DEAE-cellulose column equilibrated with 0.01M sodium phosphate buffer, pH5.5. After the column has been washed with the same buffer, the column was eluted with a zero to 0.35M sodium chloride gradient in 0.01M sodium phosphate buffer, pH5.5 (total volume 240ml in Fig. 1, 300ml in Fig. 3 and 400ml in Fig. 4). The flow rate was about 0.3g per min and 1～5 g portion was collected as indicated in each Figure. As shown in Fig. 1, two active fractions

were separated on this column, and they were termed RNase 2A and RNase 4A. They were eluted with about 0.11M and 0.21M sodium chloride, respectively. The RNase 2A fractions (No. 25 ~38) and RNase 4A fractions (No. 44~60) of Fig. 1 were pooled separately and purified as follows: both fractions were concentrated by ultrafiltration (UM-10, Amicon), and after dialyzing overnight against 0.01M sodium phosphate buffer, pH6.8, applied to a hydroxylapatite column equilibrated with 0.01M sodium phosphate buffer, pH6.8. The column was washed with the same buffer and then eluted with a linear gradient of 0.01 to 0.20M sodium phosphate buffer, pH6.8 (total volume 80 ml). The flow rate was 0.1g per min and a 1g portion was collected. The elution profiles of RNase 2A and RNase 4A were shown in Fig. 2A and 2B, respectively. Fraction No. 26

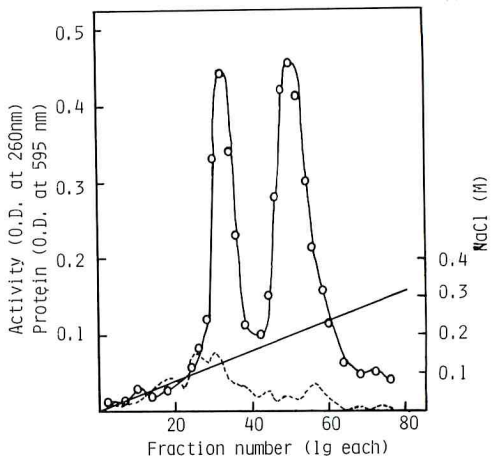

Fig. 1. Chromatography of pollen RNase A on DEAE-cellulose.

The dialyzed ammonium sulfate fraction was chromatographed on a DEAE-cellulose column (1.0×14cm) and assayed for RNase activity (○), protein concentration (···) and NaCl concentration (—).

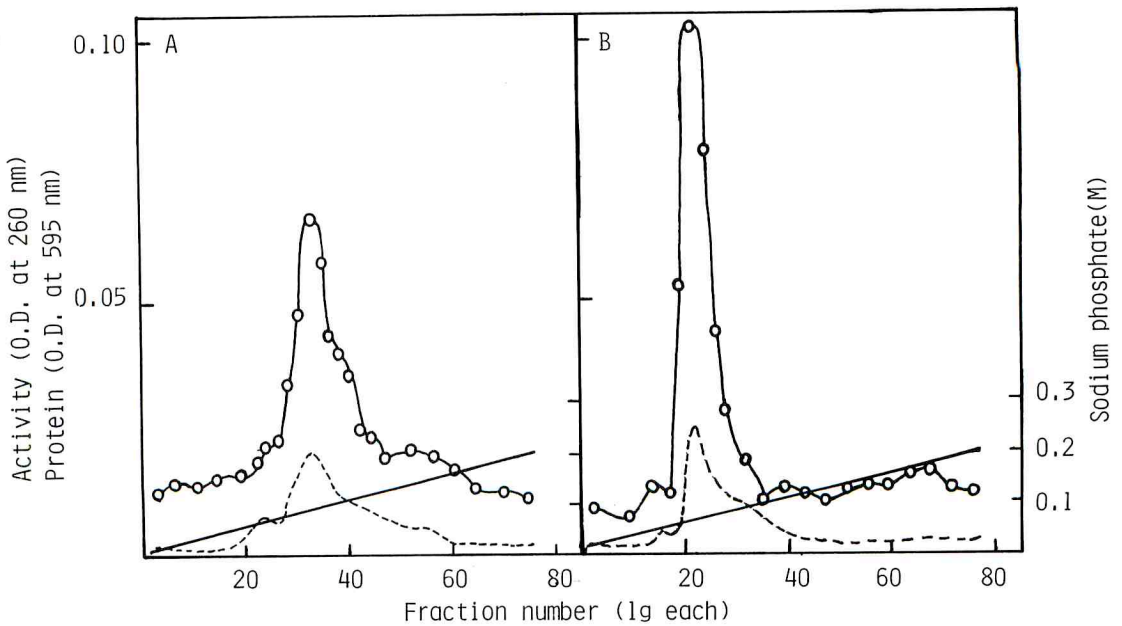

Fig. 2. Chromatography of RNase 2A (A) and RNase 4A (B) on hydroxylapatite.

The active fractions on the DEAE-cellulose column chromatography were chromatographed on a hydroxylapatite column (A, 0.7×11cm ; B, 0.7×12cm) and assayed for RNase activity (○), protein concentration (···) and sodium phosphate concentration (—).

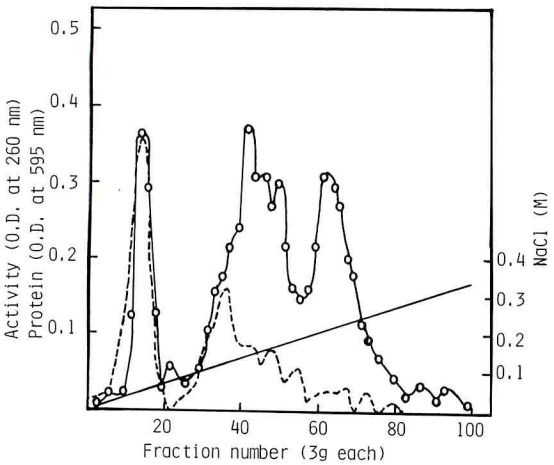

Fig. 3. Chromatography of pollen RNase B on DEAE-cellulose.

The dialyzed ammonium sulfate fraction was chromatographed on a DEAE-cellulose column (1.0×20cm). Symbols represent the same as in Fig. 1.

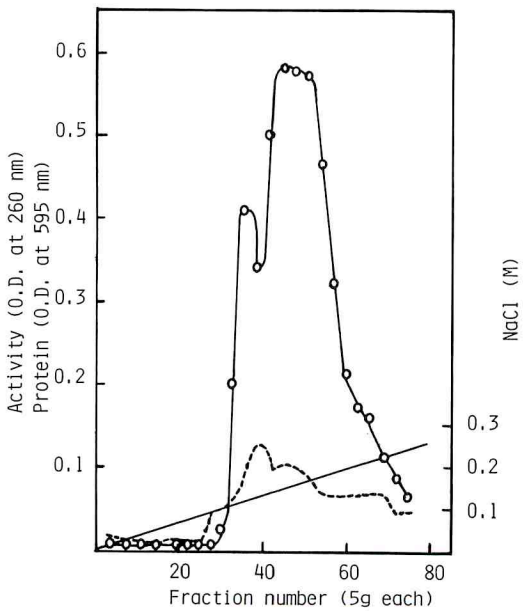

Fig 4. Resolution of RNase 2B and RNase 3B on DEAE-cellulose.

The active fractions (RNase 2B～3B) on the DEAE-cellulose column chromatography were subjected to a second DEAE-cellulose column (1.0×11cm). Symbols represent the same as in Fig. 1.

～42 of Fig. 2A and fraction No. 18～32 of Fig. 2B were packed separately in visking tubes and concentrated with a solid Sephadex G-200, and used for the following experiments.

Purification of RNase B from pine pollen

The residual fraction of the 0.5M sodium chloride extraction as described above, was suspended in 10 volumes of 0.1M phosphate buffer, pH5.5, and disrupted with a French Press at 400～600kg per cm², and stirred for 30 min. The homogenate was filtered through 4 layers of gauze and centrifuged at 17,000×g for 20 min. The supernatant obtained was filtered again through 4 layers of gauze to remove contaminated lipids. The filtrate was precipitated with 0.3 saturation of ammonium sulfate. After standing for 4 hr, the precipitate obtained by centrifugation at 17,000×g for 20 min was discarded and the supernatant obtained was brought to 0.7 saturation of ammonium sulfate, and after standing overnight, the precipitate was collected by contrifugation at 17,000×g for 20 min and dissolved in 0.01M phosphate buffer, pH5.5. The following operations were carried out as described in RNase A.

As shown in Fig. 3, three active fractions of RNase were obtained by the DEAE-cellulose column chromatography. They were termed RNase 1B, RNase 2B～3B and RNase 4B. They were eluted with about 0.05M, 0.11～0.15M and 0.21M sodium chloride, respectively. Since RNase 2B～3B were not separated well on this column, the active fractions (No. 30～54) were pooled, concentrated by ultrafiltration and dialyzed as described before, and applied to a second DEAE-cellulose column equilibrated with 0.01M phosphate buffer, pH5.5. The column was eluted with a zero to 0.25M sodium chloride gradient in 0.01M phosphate buffer, pH5.5 (total volume 400ml). As

shown in Fig. 4, two active fractions were partially separated on the second DEAE-cellulose column, and from now they were termed RNase 2B and RNase 3B. They were eluted with about 0.11M and 0.15M sodium chloride, respectively. The RNase 1B fractions (No. 10～20) of Fig. 3, the RNase 2B fractions (No. 32～39) of Fig. 4, the RNase 3B fractions (No. 42～60) of Fig. 4 and the RNase 4B fractions (No. 60～78) of Fig. 3 were pooled separately and concentrated by ultrafiltration (UM-10, Amicon), and after dialyzing against 0.01M phoshate buffer, pH6.8, applied to a hydroxylapatite column. The column was eluted as described before. The elution profiles of RNase 1B, 2B, 3B and 4B were shown in Fig. 5A, 5B, 5C and 5D, respectively. Each RNase was pooled, and after concentrating with a solid Sephadex G-200, was used for the following experiments. Each RNase fraction thus obtained was virtually free from phosphodiesterase and phosphomonoesterase using bis p-nitrophenyl phosphate and p-nitrophenyl phosphate as substrates, respectively.

All operations for the preparation of RNase were carried out at 0～5°C unless stated otherwise.

The outline and the result of purification of RNase A and RNase B are summarized in Table 1. The specific activity of RNase 2A, 4A, 1B, 2B, 3B and 4B increased 18.2, 30.7, 23.3, 48.9, 57.5 and 109.8-fold, respectively. The yields of RNase 2A, 4A, 1B, 2B, 3B and 4B were 2,8, 4.7, 1.9, 2.9, 5.2 and 15.9%, respectively.

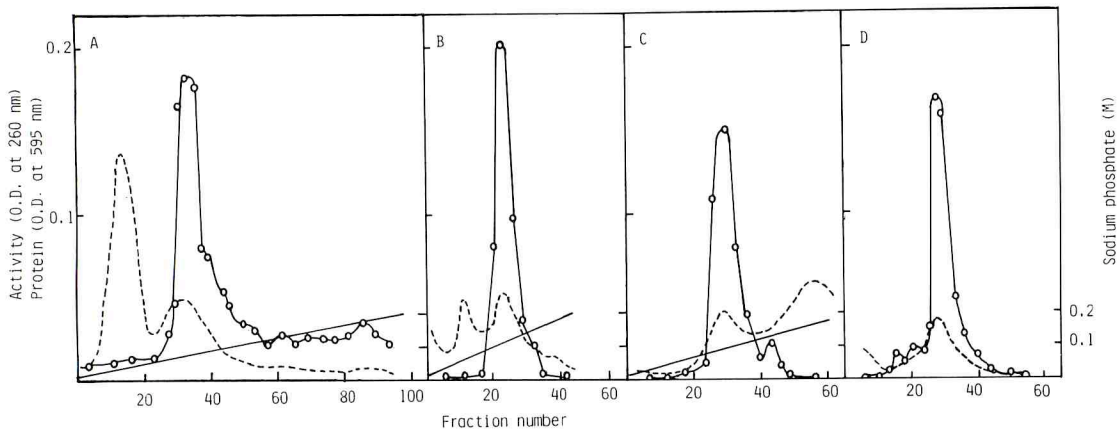

Fig. 5. Chromatography of RNase 1B(A), RNase 2B(B), RNase 3B(C) and RNase 4B(D) on hydroxylapatite.

The active fractions on the DEAE-cellulose column chromatography were chromatographed on a hydroxylapatite column (A and D, 0.7×11cm ; B, 0.9×14cm ; C, 0.9×13cm). One gram portion for RNase 1B and RNase 4B, 5g for RNase 2B and 3g for RNase 3B were collected. Symbols represent the same as in Fig. 2.

Table 1. Purification of pine pollen RNases

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	Yield (%)
RNase A						
Crude extract	670	19.4	1,137	58.6	1.0	100
70%$(NH_4)_2SO_4$	14	5.0	699	139.8	2.4	61.5
DEAE-cellulose						
RNase 2 A	4.8	0.2	120	600.0	9.8	10.6
RNase 4 A	4.8	0.1	141	1,410.0	24.1	12.9
Hydroxylapatite						
RNase 2 A	2.2	0.03	32	1,066.7	18.2	2.8
RNase 4 A	2.2	0.03	54	1,800.0	30.7	4.7
RNase B						
Crude extract	920	165.6	12,221	73.8	1.0	100
30～70%$(NH_4)_2SO_4$	26	67.0	8,029	119,8	1.6	65.7
DEAE cellulose						
RNase 1 B	6.0	8.0	1,230	153.8	2.1	10.0
RNase 2 B～3 B*	5.2	4.0	1,118	279.5	3.8	9.1
RNase 4 B	4.1	3.0	2,320	773.3	11.0	18.9
Hydroxylapatite						
RNase 1 B	1.4	0.14	241	1,721.4	23.3	1.9
RNase 2 B	1.0	0.10	361	3,610.0	48.9	2.9
RNase 3 B	1.5	0.15	637	4,246.7	57.5	5.2
RNase 4 B	1.2	0.24	1,945	8,104.2	109.8	15.9

* Data obtained from second DEAE cellulose column chnomatography

Properties of RNase A and RNase B

Molecular weight. From the results of Fig. 6, the molecular weight of each RNase was estimated as follows: RNase 2A, RNase 2B and RNase 3B, 45,000; RNase 1B, 42,000; RNase 4A, 28,000; RNAse 4B, 26,000.

Effect of pH on the activity. The effect of pH on RNase 2A activity was examined by the standard assay system in which pH values were varied as indicated in Fig. 7. RNase 2A had an optimum pH of 5.5. Other pine pollen RNases exhibited also an optimum pH of about 5.5.

pH stability. RNase 2A in 0.025M buffer was kept overnight at different pH values as indicated in Fig. 8 at 4°C and RNase 2A activity was assayed. RNase 2A was stable in the ranges of pH5.5～10.0. Other pine pollen RNases were also stable in the same ranges of pH.

Thermal stability. RNase 2A in 0.025M phosphate buffer, pH5.5, was heated for 5 min in a water bath at different temperatures as indicated in Fig. 9, and cooled immediately in ice water. RNase 2A was stable up to 50°C. Other pine pollen RNases were also stable up to 50°C, except that RNase 3B was stable up to 70°C.

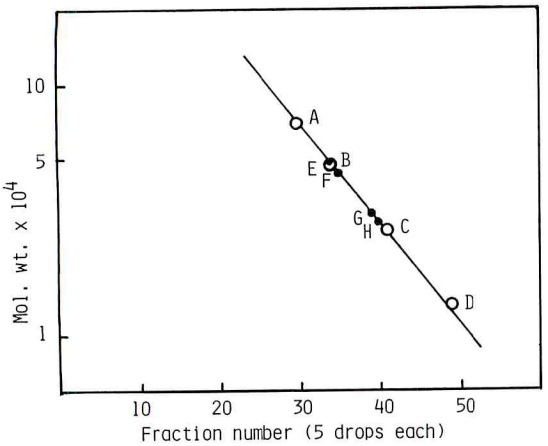

Fig. 6. Molecular weight estimation of pollen RNases by gel filtration on Sephadex G-100.

Molecular weights of the standard proteins and RNases used were as follows : A, bovine serum albumin, 68,000 ; B, ovalbumin, 45,000 ; C, chymotrypsinogen A, 25,000 ; D, cytochrome c, 12,500 ; E, RNase 2A, 2B and 3B ; F, RNase 1B ; G, RNase 4A ; H, RNase 4B.

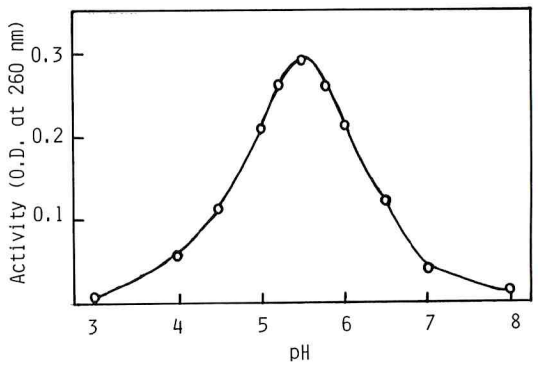

Fig. 7. Effect of pH on the activity of RNase 2A.

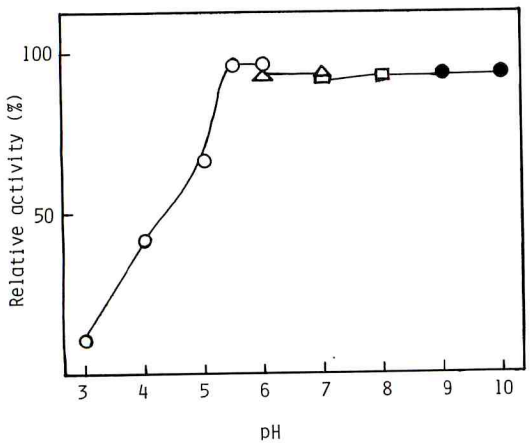

Fig. 8. Effect of pH on the stability of RNase 2A.

The buffers used were sodium acetate-acetate (○), Tris-acetate (△), Tris-HCl (□) and borate-carbonate (●).

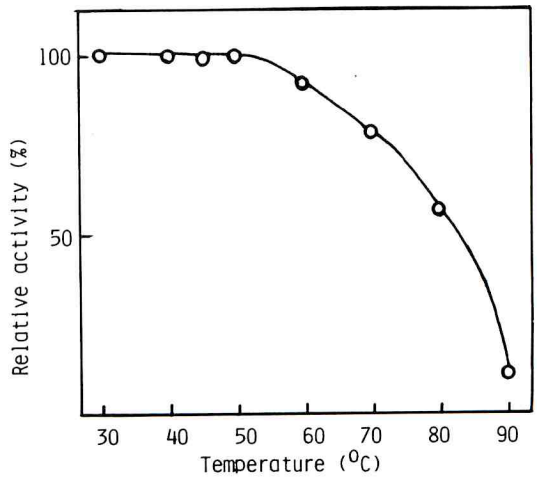

Fig. 9. Effect of heating on the activity of RNase 2A.

Effect of some reagents. As shown in Table 2, RNase 4A and RNase 4B were slightly activated by o-phenanthroline at the concentration of 1mM. Ethylenediaminetetraacetic acid (EDTA) inhibited RNase 1B, 2B and 3B. p-Chloromercuribenzoate (PCMB) inhibited RNase 2A and inhibited slightly all other RNases. o-Phenanthroline inhibited RNase 2A and 1B.

Substrate specificity. As shown in Table 3, RNase 1B, 2B, 3B and 4B could hydrolyze poly(U). RNase 2A, 1B, 2B and 3B hydrolyzed poly(A) more easily than poly(U), but, all pine pollen RNases had little or no activity when poly(C) or DNA was used as a substrate.

Table 2. Effect of some reagents on the activity of pine pollen RNases

Reagent	Relative activity (%)					
	RNase					
	2A	4A	1B	2B	3B	4B
None	100	100	100	100	100	100
EDTA	85	118	46	41	47	109
PCMB	46	65	80	76	85	70
o-Phenanthroline	51	172	31	68	85	135
Oxalic acid	89	82	12	22	29	61

Hydrolysis of the yeast RNA was measured in the standard assay system except that 1 mM of reagents, 0.3mM in the case of PCMB, was added to the reaction mixture.

Table 3. Substrate specificity of pine pollen RNases

Substrate	Relative activity (%)					
	RNase					
	2A	4A	1B	2B	3B	4B
Yeast RNA	100	100	100	100	100	100
Poly(A)	54	3	81	55	57	3
Poly(U)	1	1	56	21	21	22
Poly(C)	1	0	0	0	0	1
Native DNA	0	0	0	0	0	0
Denatured DNA	0	0	0	0	0	0

Hydrolysis of the polynucleotides and herring sperm DNA was measured in the standard assay system except that 0.5% polynucleotide, native- or denatured DNA was added to the reaction mixture. Denatured DNA was prepared by heating a solution of native DNA (5mg/ml) at 100°C for 20 min and then cooled rapidly in ice. The amount of enzyme that causes an increase in absorbance of 0.15 at 260nm under the standard assay system was used.

Test of endo- or exonuclease activity. To distinguish between exo- or endonucleolytic modes of action, aliquots of poly(U) or poly(C) hydrolyzed to different extents were analyzed by a Sephadex G-75 gel filtration. As shown in Fig. 10, the chromatographic profiles of the poly(U) digests with RNase 2A showed a continuous distribution of poly(U) fragments. All the other RNases also showed similar elution profiles. When poly(C) was used as a substrate, however, only the hydrolyzate by RNase 2A showed similar elution profiles to that of Fig. 10. These data show that all RNases are endonucleases.

Base specificity. To determine the base specificity of pollen RNase for yeast RNA, the digests of yeast RNA with pollen RNase, or the hydrolysis products of these digests with bull semen 5′-nucleotidase, were identified by HPLC. Figure 11 shows an elution profile of the digests of yeast RNA with RNase 3B on HPLC. The main three peaks, 1, 2 and 3, corresponded to 2′-AMP or 5′-AMP, 5′-UMP, and 2′, 3′-UMP or 5′-GMP, respectively (Fig.11A). As shown in Fig. 11B, these three peaks disappeared completely after the 5′-nucleotidase treatment. From these renults peaks 1,2 and 3 in Fig. 11A were identified to be 5′-AMP, 5′-UMP and 5′-GMP, respectively. Figure 11A shows also that the digested rate of these three 5′-nucleotides from yeast RNA with RNase 3B, was in order 5′-AMP>5′-GMP>5′-UMP. The elution profiles of digests of yeast RNA with RNase 2A, 1B and 2B on HPLC showed similar results as shown in Fig. 11. Figure 12

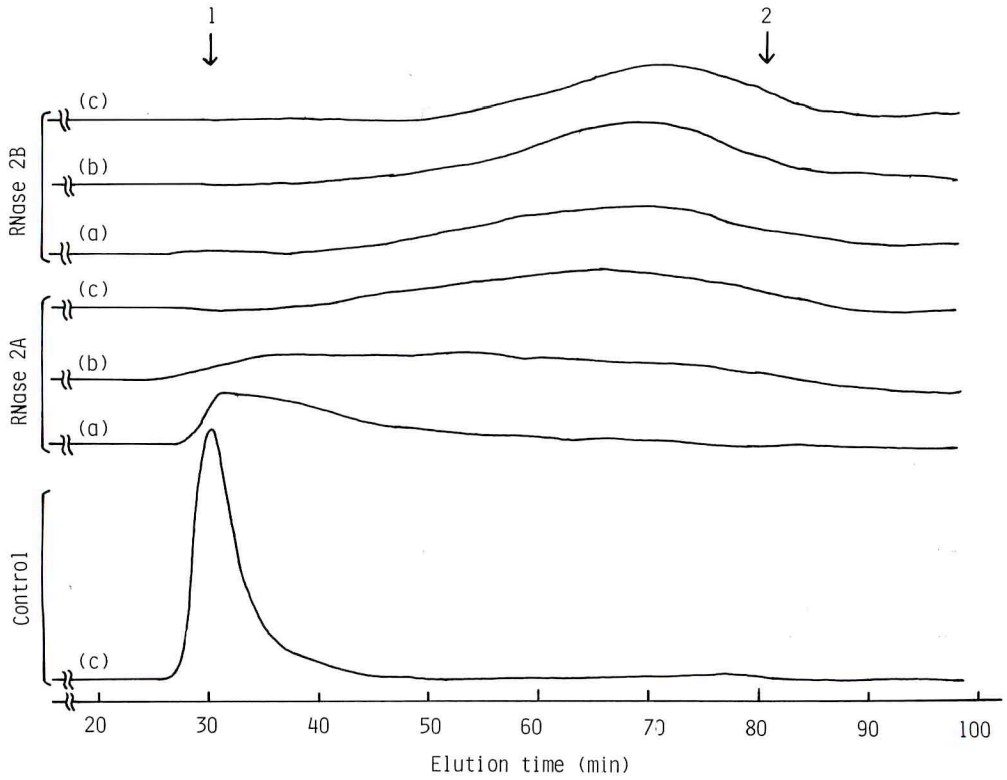

Fig. 10. Chromatography of the digests of poly(U) with RNase 2A and RNase 2B on Sephadex G-75.
Poly(U) hydrolyzed with RNase 2A or RNase 2B for the following times at 37°C was subjected to a Sephadex G-75 column chromatography : (a) 3 hr ; (b) 6 hr ; (c) 18 hr. Arrows 1 and 2 indicate the elution time of poly(U) and of UMP, respectively.

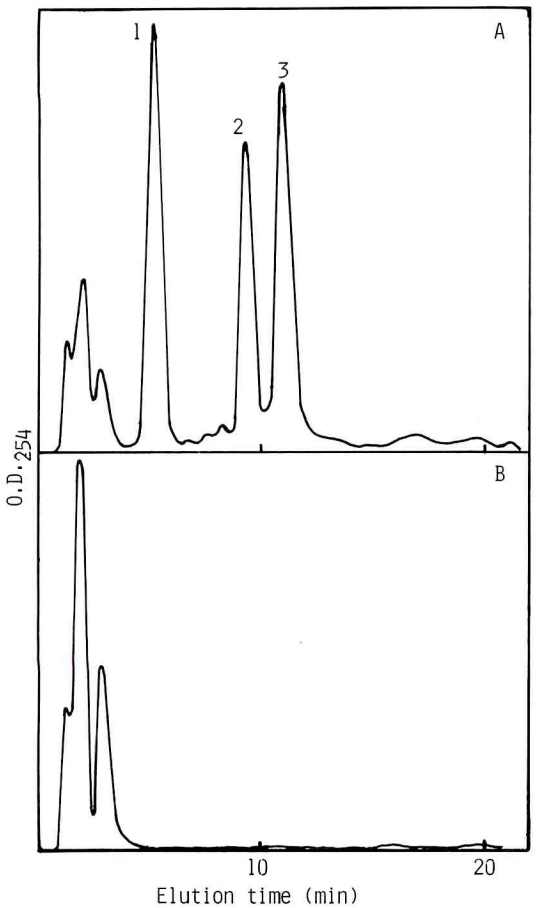

Fig. 11. HPLC analysis of the digests of yeast RNA with RNase 3B.
A, digests of yeast RNA with RNase 3B ; B, hydrolysis products of the above digests with bull semen 5′-nucleotidase.

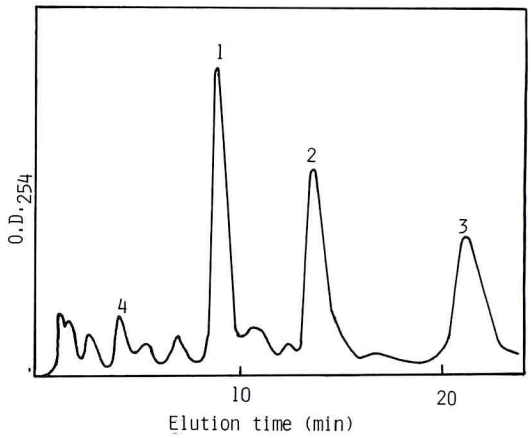

Fig. 12. HPLC analysis of the digests of yeast RNA with RNase 4A.

shows an elution profile of the digests of yeast RNA with RNase 4A on HPLC. The main three peaks, 1, 2 and 3, were identified to be 2′, 3′-cAMP, 2′, 3′-cUMP and 2′, 3′-cGMP, respectively. Peak 4 corresponding to 2′, 3′-cCMP was very small. Figure 12 shows also that the digested rate of these three 2′, 3′-cyclic nucleotides from yeast RNA with RNase 4A, was in order 2′, 3′-cAMP＞2′, 3′-cUMP≒2′, 3′-cGMP. The main three peaks shown in Fig. 12 did not disappear after the 5′-nucleotidase treatement. The elution profile of digests of yeast RNA with RNase 4B on HPLC showed the liberation of 2′, 3′-cCMP, 2′, 3′-cAMP, 2′, 3′-cUMP and 2′, 3′-cGMP.

Discussin

As described above, multiple forms of RNase were detected in the pine pollen. Among these RNases, RNase 2A and RNase 4A may be associated with the intine, the inner cellulosic part of the cell wall of pine pollen, and RNase 1B, 2B 3B and 4B may be localized in the cytoplasm.

Although the molecular weights of RNase 4A and RNase 4B are similar to those of other higher plant RNases,[36] such as corn seed (23,000)[37] and rye germ (28,500);[38] the molecular weights of RNase 2A, 1B 2B and 3B are higher than those of higher plants. The optimum pH of RNase 2A and other pine pollen RNases was very close to the reported values of 5.5 for pea leaves[39] and rape pollen,[31] 5.1 for tobacco leaves,[40] 5.0～5.5 for mung bean sprouts,[41~44] 5.6 for cucumber seedlings,[45] 5.7 for wheat germ[46] and Japanese cycad pollen[29] and 5.8 for rye germ[38]. The pH stability of all pine pollen RNases was similar

to that of RNase from Japanese cycad seed endosperm[47] and mung bean sprouts;[48] All pine pollen RNases were not so thermally stable as tobacco leaf RNase;[40] spinach leaf RNase [49] and cucumber seedlings RNase.[45]

Plant RNase splits synthetic polynucleotides. In the cases of wheat germ RNase [46] and barley seed RNase;[50] the relative rate of degradation was poly (U)>poly(A)>poly(C). Rye germ RNase[38] hydrolyzed homopolymers in the following order : poly(A)>poly(C)>poly(U)>poly(G). All pine pollen RNases except RNase 4B split homopolymers in the following order : poly(A)>poly(U)>poly(C). As shown in Fig. 10, the chromatographic profiles of the poly(U) digests with RNase 2A, showed a continuous distribution of poly(U) fragments. All the other pine pollen RNases showed similar profiles. These results suggest that all pine pollen RNases have an endonucleolytic mode of action.

Plant RNases, such as those derived from rye grass;[51] spinach leaves;[49] mung bean sprouts;[41~43] corn roots;[52] Japanese cycad seed endosperm;[53] ginkgo seeds;[54] tea leaves;[55] Japanese cycad pollen[29] and a number of other plant species;[36] hydrolyze RNA, yielding 2′, 3′-cyclic mononucleotides. RNase 4A and RNase 4B were found similar type to these enzymes. On the other hand, RNase 2A, 1B, 2B and 3B released 5′-mononucleotides from RNA at the following rate : 5′-AMP>5′-GMP>5′-UMP. In this regard these pine pollen RNases were similar to the RNases obtained from mung bean sprouts[44] and *Lactobacillus casei*.[56]

Multiple forms of plasnt RNases have been demonstrated in mung bean sprouts;[41] Japanese cycad seed embryo;[53] ginkgo seeds;[54] tea leaves;[55] bean roots;[57] barley seeds;[50] rice bran[58] and other higher plant species;[36] although the biological significance of the presence of RNases in multiple forms is unknown. RNase 2A, 1B, 2B and 3B have similar molecular weight and mode of action, and other properties were nearly the same. However, some differences are observed between RNase 2A, which may be associated with the cell-surface, and RNase 2B, which may be localized in the cytoplasm, with respect to hydrolysis rate of poly(U) and inhibitory effects of EDTA, PCMB and oxalic acid (Fig. 10 and Table 2 and 3). RNase 4A, which may be associated with the cell-surface, and RNase 4B, which may be localized in the cytoplasm, have similar mode of action, and other properties were nearly the same, however, some differences are observed between both RNases with respect to molecular weight and hydrolysis rate of poly(U) (Fig. 6 and Table 3). Therefore, we concluded that there are two groups of RNases in pine pollen. That is, 2′, 3′-cyclic mononucleotide forming group and 5′-nucleotide forming group. RNase 4A and RNase 4B are the former group, and RNase 2A, 1B, 2B and 3B are the latter group, respectively. Moreover, all of the RNases have slightly different properties within each groups.

Although the real functions of each RNase are still no clear, it is possible that multiple forms of RNase A and RNase B may play a crucial role in the process of germination as relative amounts of each RNase change greatly during the process of germination (to be published elsewhere).

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要　約

完熟アカマツ花粉を用い、0.5 M 塩化ナトリウム溶液で振とうすることによって溶出するリボヌクレアーゼ（RNase A）と、塩化ナトリウム溶液処理後に破砕花粉から溶出するリボクレアーゼ(RNase B)を検討し次の結果を得た。

1．各種クロマトグラフィーにより RNase 2A、4A、1B、2B、3B、4B を分離したが、分子量は RNase2A、2B、3B は 45,000、RNase 1B は 42,000、RNase 4A は 28,000、RNase 4B は 26,000 であった。各 RNase とも至適 pH はほぼ 5.5、pH5.5～10.0 で安定で、50°C 5 分間加熱まで安定(RNase 3B は 70°C まで安定）であった。

2．各 RNase が poly((U)に対してエンドヌクレアーゼとして作用した。

3．RNase 2A が若干 poly(C)を分解したが、その他すべての RNase が poly(C)および DNA(ニシン精液）を分解しなかった。

4．RNase2A、1B、2B、3B は酵母 RNA を分解して 5′-AMP、5′-UMP、5′-GMP を生成し、RNase 4A、4B は 2′, 3′-サイクリック AMP、2′, 3′-サイクリック UMP、2′, 3′-サイクリック GMP、2′,3′-サイクリック CMP（少量）を生じた。