Purification and characterization of ribonucleases from human seminal plasma

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Four ribonucleases (RNAases I–IV) have been purified to homogeneity from human seminal plasma by precipitation with 40–75%-satd. $(\text{NH}_4)_2\text{SO}_4$, followed by chromatographies on concanavalin A–Sephadex 4B, DEAE-cellulose phosphocellulose, agarose–5'-(4-aminophenylphospho)uridine 2' (3')-phosphate (RNAase affinity column) and Sephadex G-75 or G-100. The homogeneity of these RNAases was confirmed by polyacrylamide-gel electrophoresis. M_r values for these purified RNAases were 78 000, 16 000, 13 300 and 5000 as estimated by gel filtration. Enzyme activities of RNAases I, III and IV were inhibited by Mn^{2+} , Zn^{2+} and Cu^{2+} and activated by Na^+ , K^+ , Ba^{2+} , Mg^{2+} , Fe^{2+} and EDTA, whereas that of RNAase II was inhibited by Ba^{2+} , Mg^{2+} , Fe^{2+} , Mn^{2+} , Zn^{2+} and Cu^{2+} and activated by Na^+ , K^+ and EDTA. RNAases I, II and IV demonstrated a higher affinity for poly(C) and poly(U) or yeast RNA, whereas RNAase III preferentially hydrolysed poly(U) over poly(C) and yeast RNA. In the presence of 5 mM-spermine, RNAase I was dissociated to a low- M_r (5000) enzyme with an increase in total RNAase enzymic activity. Xenoantiserum to each RNAase was raised and evaluated by immunoprecipitation and immunohistochemical methods. Anti-(seminal RNAase III) antiserum showed no immunological cross-reaction with RNAases of other human origin, whereas anti-(seminal RNAase I), -(RNAase II) and -(RNAase IV) antisera exhibited indistinguishable immunological reactions with serum RNAase and other human RNAases, except that anti-(seminal RNAase I) and -(RNAase antisera IV) did not react with pancreatic RNAases. Seminal RNAases I and IV were identical immunologically as shown by anti-(RNAase I) and anti-(RNAase IV) in immunodiffusion. Immunohistochemical study revealed that, among human tissues examined, only prostate expressed seminal RNAase III. These results suggested that human seminal RNAase I may be an aggregated molecule of RNAase IV and that seminal RNAases II and IV are similar to serum RNAases, whereas seminal RNAase III is a prostate-specific enzyme.

Two types of RNAase activity have been recognized to exist in human tissues and fluids: acid and alkaline (Ressler *et al.*, 1966). They differ from each other in catalytic characteristics and antigenic specificities (Sierakowska & Shugar, 1977). Although purification and characterization of RNAases from human organs such as the liver (Frank & Levy, 1976), spleen (Neuwelt *et al.*, 1976), pancreas (Neuwelt *et al.*, 1977), serum (Shmukler *et al.*, 1975;

Akagi *et al.*, 1976), urine (Rabin *et al.*, 1977; Yamanaka *et al.*, 1977) and cerebrospinal fluid (Rabin *et al.*, 1977) have been reported, the correlation of structure with function and property in these RNAases is still unclear, and even the changes in RNAase activities under normal physiological or pathological conditions are poorly understood.

Recently, variation in the levels of RNAase activity in human serum and urine was suggested to be indicative of a disease state, including cancer (Neuwelt *et al.*, 1977; Shield *et al.*, 1977; Reddi,

Abbreviations used: RNAase, ribonuclease; NaDodSO₄, sodium dodecyl sulphate.

1978). We have also detected elevated serum RNAase activity in patients with prostatic cancer (Chu *et al.*, 1977). Since only the activity of circulating enzyme was measured in our study, further assessment of the RNAase is essential. A limited source of human prostate has prompted us to the use of seminal plasma, as secretion of prostate gland contributes a major portion of seminal plasma (Eliasson, 1973). The present paper describes the purification of a total of four RNAases from human seminal plasma. Biochemical and immunological characterizations of the purified enzymes are also described.

Materials and methods

RNAase assay

The standard assay of RNAase activity was performed by a modified method of Akagi *et al.* (1978) in a reaction mixture (0.11 ml) containing 0.1 ml of yeast RNA (3 mg/ml, or synthetic polyribonucleotide) in 0.1 M-sodium phosphate, pH 7.0, and 0.01 ml of enzyme (1–5 μ g). The amount of acid-soluble nucleotides produced was determined at 260 nm (Kalnitsky *et al.*, 1959). One unit of RNAase activity was defined as that which produced an absorbance of 0.01.

Purification of human seminal RNAases

The entire isolation procedure was carried out at 4°C as follows and is summarized in Table 1.

Step 1. Crude RNAases were extracted from human seminal plasma, upon removal of sperms, by 40–75% satd. $(\text{NH}_4)_2\text{SO}_4$ and the precipitate was dissolved in starting buffer, pH 5.0 (1 mM each for CaCl_2 , MgCl_2 and MnCl_2 , and 0.01 M each for NaCl and sodium acetate). The sample was applied to a concanavalin A-Sepharose 4B column (2 cm $\times$ 40 cm; concanavalin A column) (Lee *et al.*, 1978). The non-binding proteins exhibiting RNAase activity were eluted with starting buffer, pH 5.0, and were processed further as described in Step 2. RNAases and other glycoproteins which bound to the concanavalin A column, were eluted with a linear concentration gradient of 0.1–0.5 M- α -methyl D-mannopyranoside. Fractions (3 ml/tube) were collected and measured for both RNAase activity and protein (280 nm), and processed further as described in Step 3.

Step 2. Fractions which did not bind to concanavalin A and exhibited RNAase activity were applied to an agarose-5'-(4-aminophenylphospho)uridine 2'(3')-phosphate column (RNAase affinity column; 1.5 cm $\times$ 10 cm; Wilchek & Gorecki, 1969).

Table 1. Preparation of homogeneous ribonucleases from human seminal plasma

Procedure	Total protein* (mg)	Total enzyme† activity (units)	Specific activity (units/mg)	Relative activity	Recovery (%)
Human seminal plasma	12 150	5675	0.5	1.0	100§
$(\text{NH}_4)_2\text{SO}_4$ precipitate	1410	3405	2.4	5.1	60.0§
Concanavalin A- Sepharose 4B					
RNAases I and II	300	1348	4.5	9.6	23.8
RNAases III and IV	225	1925	8.6	17.2	33.9¶
Cellulose phosphate					
RNAases I and II	10.6	908	86.0	183.0	16.0
DEAE-cellulose					
RNAase III	62.4	965	15.5	32.9	17.0
RNAase IV	92.4	865	9.4	19.9	15.2
Affinity column‡					
RNAases I and II	1.46	388	265.8	565.4	6.8
RNAase III	7.40	759	102.6	218.2	13.3
RNAase IV	5.40	659	122.1	259.7	11.6
Sephadex G-75					
RNAase I	0.63	185	290.5	618.0	3.2
RNAase II	0.15	195	1300.0	2766.0	3.4
RNAase III	0.60	514	856.7	1822.7	9.0
RNAase IV	0.20	162	813.0	1729.8	2.8
Sephadex G-100					
RNAase I	0.40	175	437.5	930.9	3.0

* Protein concentration was determined by the method of Lowry *et al.* (1953).

† Enzyme activity was determined by the modified method of Akagi *et al.* (1978).

‡ RNAase affinity column: agarose(5'-(4-aminophenylphospho)uridine 2'(3')-phosphate column.

§ Combined recovery of RNAases I–IV.

|| Combined recovery of RNAases I and II.

¶ Combined recovery of RNAases III and IV.

After washing the column with 0.02 M-sodium acetate, pH 5.0, RNAases were eluted with 4 M-NaCl. Fractions containing RNAases (designated I and II) were further applied to a phosphocellulose column (1.5 cm × 40 cm; Akagi *et al.*, 1976) and eluted with a linear concentration gradient of 0.1–0.5 M-NaCl in 0.01 M-sodium phosphate, pH 7.5. The RNAase-containing fractions were pooled and concentrated. RNAases I and II were further purified to homogeneity on columns of Sephadex G-75 (2 cm × 96 cm) and G-100 (2 cm × 95 cm).

Step 3. Fractions that bound to concanavalin A and exhibited RNAase activity were applied to a column (2 cm × 46 cm) of DEAE-cellulose. Two RNAase activity peaks (designated III and IV) were eluted from the column by a convex NaCl and pH gradient (0.01 M-sodium phosphate, pH 7.0–4.5, containing 0–0.5 M-NaCl). Fractions containing RNAases III and IV were pooled separately and further passed through the RNAase affinity column for additional purification as described above. RNAases III and IV were purified to homogeneity by a final gel filtration on a column (2 cm × 96 cm) of Sephadex G-75.

Polyacrylamide-gel electrophoresis

Disc polyacrylamide-gel electrophoresis was performed by the method of Davis (1967) in a standard 7.5% (w/v) polyacrylamide gel. NaDodSO₄/polyacrylamide gel electrophoresis was performed in 7.5% polyacrylamide gel by a modified method of Weber & Osborn (1969). The protein was denatured before electrophoresis by incubating it with 0.05 M-phosphate buffer, pH 7.0, containing 1% NaDodSO₄, 1% mercaptoethanol and 5 M-urea at 37°C for 2 h.

Physicochemical study

The M_r values for these purified RNAases were determined by gel filtration on calibrated columns of Sephadex G-75 (2 cm × 96 cm) and G-100 (2 cm × 95 cm) with 0.02 M-sodium phosphate, pH 7.5. Sedimentation coefficients were determined as described previously (Loor *et al.*, 1981). After centrifugation, RNAase activity and protein of each fraction were determined by the RNAase assay described above and the Lowry method (Lowry *et al.*, 1951) respectively, and localized by reference marker proteins. Purified RNAase was subjected to electrophoresis as reported previously (Lee *et al.*, 1980a), a 100-ml LKB electrophoresis column being used.

Effect of spermine on RNAase I

Seminal RNAase I (1 mg/ml) was mixed with 5 mM-spermine and allowed to stand at room temperature for 10 min and then passed through a Sephadex G-75 column (2 cm × 96 cm). Enzyme was

eluted with 0.01 M-sodium phosphate buffer, pH 7.5. Fractions were collected and measured for both RNAase activity and protein as described above.

Amino acid analysis

The purified RNAase was hydrolysed in 6 M-HCl for 24 h at 110°C in a sealed evacuated tube. Amino acid analysis was performed on a Dionex model D-500 automatic amino acid analyser (Chang *et al.*, 1979).

Immunological methods

Anti-RNAase antisera were raised as described by Chu *et al.* (1978). Ouchterlony gel immunodiffusion and immunoelectrophoresis were performed as described previously by Lee *et al.* (1980a). Immunohistochemical staining of RNAase was performed by the method of Li *et al.* (1980). A conventionally prepared formalin-fixed paraffin-embedded block was used. The negative control groups included those treated with normal rabbit immunoglobulin or phosphate-buffered saline, pH 7.2, under identical conditions.

Results

Purification

Four RNAases from human seminal plasma were purified to homogeneity by the procedure described. The homogeneity of the purified enzymes was confirmed by disc polyacrylamide-gel electrophoresis, which demonstrated a single band of protein for each RNAase with and without NaDodSO₄ (Fig. 1), with the exception of RNAase I. RNAase I showed a single protein band without NaDodSO₄ and a major band at M_r 78 000 and a minor band at M_r 5000 (less than 10% of total protein) upon NaDodSO₄ treatment. A typical purification scheme is summarized in Table 1.

Biochemical characterization

These four human seminal RNAases were characterized and the results are summarized in Tables 2–5.

The M_r values for these four purified RNAases, as estimated by Sephadex G-75 and G-100 chromatography, were: 78 000 for RNAase I; 16 000 for RNAase II; 13 000 for RNAase III; and 5000 for RNAase IV. Sucrose-gradient centrifugation showed sedimentation coefficients of 4.75 S, 2.30 S and 1.85 S for RNAases I, II and III respectively. An accurate value was not experimentally possible for RNAase IV. Preparative isoelectric focusing revealed that RNAase III was a strongly basic protein with a single pI value of 11.0 as compared with a pI value of 9.9 for RNAase II and an acidic pI value of 4.8 for RNAase IV (Table 2).

The hydrolytic activity of human seminal RNAases at various (4.0–9.0) pH values was measured with yeast RNA as the substrate in 0.1 M-sodium phosphate buffer containing 0.15 M-NaCl. The pH optima of these four RNAases were 8.0 (RNAase I), 8.5 (RNAase II) and 7.5 (RNAases III

Fig. 1. Disc polyacrylamide-gel electrophoresis of RNAases

(a) RNAases I, II and III (25 μ g of each) in 7.5% (w/v) polyacrylamide gel in β -alanine/acetic acid buffer, pH 4.3. Protein migrated to the cathode (bottom). (b) RNAase IV (25 μ g) in 7.5% polyacrylamide gel in glycine/Tris buffer, pH 8.5, and migrated toward the anode (bottom). Electrophoresis was performed at 4°C with a constant current of 4 mA/tube for 1 h. Protein bands were stained with Coomassie Brilliant Blue G-250 in 3.5% (w/v) HClO_4 .

and IV). The hydrolytic activity of these RNAases depends on the type of buffer used. Enzyme activity in Tris/HCl buffer provided an identical optimal pH for all RNAases, with a 2-fold greater enzyme activity than that detected in phosphate buffer.

All RNAases were adjusted to 0.2 mg/ml in 0.02 M-sodium phosphate buffer, pH 7.5, for the thermostability study. The remaining enzyme activity of each RNAase preparation after heating at 55°C for 30 min is presented in Table 2.

The effect of various ionic species and chemicals on RNAase enzymic activity was measured by their hydrolysis of yeast RNA (3 mg/ml in 0.1 M-sodium phosphate buffer) at their optimal pH values. RNAases I and IV were activated by Na^+ , K^+ , Ca^{2+} , Ba^{2+} , Mg^{2+} , Fe^{2+} and EDTA, but inhibited by Mn^{2+} , Zn^{2+} and Cu^{2+} , whereas RNAase II was activated by Na^+ , K^+ and EDTA and inhibited by Ca^{2+} , Ba^{2+} , Mg^{2+} , Fe^{2+} , Mn^{2+} , Zn^{2+} and Cu^{2+} (RNAase III also was activated by Na^+ , K^+ , Mg^{2+} , Ba^{2+} , Fe^{2+} and EDTA, and inhibited by Ca^{2+} , Mn^{2+} , Zn^{2+} , and Cu^{2+}). The bivalent cations Mn^{2+} , Zn^{2+} and Cu^{2+} showed a significant inhibitory action on these four RNAases, whereas EDTA and the univalent cations, Na^+ and K^+ , were effective activators.

Relative specificity of these RNAases toward synthetic polyribonucleotides and yeast RNA is detailed in Table 3 and summarized in Table 2. RNAase III degraded poly(U) more than poly(C) and yeast RNA to form acid-soluble nucleotides (Table 3), whereas RNAases I, II and IV preferentially hydrolysed poly(C) rather than poly(U) and yeast RNA. These four RNAases were unable to degrade poly(A) or poly(G).

K_m and V_{max} for hydrolysis of poly(U) and poly(C) by these RNAases were evaluated. K_m and V_{max} values, as calculated by the method of Lineweaver & Burk (1934), are shown in Table 4. RNAases I, II and IV had a higher affinity towards

Table 2. Summary of biochemical properties of human seminal RNAases*

	RNAase ... I	II	III	IV
M_r	78 000	16 000	13 000	5000
Sedimentation coefficient (S)	4.75	2.30	1.85	N.D.†
Optimal pH	8.0	8.5	7.5	7.5
Isoelectric point (pI)	N.D.	9.9	11.0	4.8
Thermostability (%)†	90	95	75	80
Base-specificity polynucleotide	Poly(C)	Poly(C)	Poly(U)	Poly(C)
K_m (M)‡	8×10^{-8}	1.1×10^{-8}	2.4×10^{-9}	2.5×10^{-8}
V_{max} §	33.3	5.3	1.1	8.3

* Values were averages for duplicate experiments.

† Thermostability was determined by heating each RNAase at 55°C for 30 min (Bardon *et al.*, 1976). Values were calculated by comparing the remaining enzyme activity with an untreated control.

‡ Calculated by a Lineweaver–Burk (1934) plot of $1/\text{velocity}$ versus $1/[\text{substrate}]$, using poly(U) as the substrate.

§ Expressed in $\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (Lineweaver & Burk, 1934).

|| ND, Not determined.

Table 3. *Hydrolysis of synthetic polyribonucleotides by human seminal ribonucleases*

The experiments were performed at pH 8.9 (RNAase I), 8.5 (RNAase II) and 7.5 (RNAase III and IV). The reaction mixture consisted of 100 μ l of substrate (1 mg/ml) in 0.1 M-sodium phosphate and RNAase (5 μ g/10 μ l). RNAase activity was measured as described in the Materials and methods section. Values were averages for duplicate experiments.

Substrate	RNAase	...	Enzyme activity (A_{260})			
			I	II	III	IV
Poly(U)			0.565	0.524	0.657	0.450
Poly(C)			0.667	0.694	0.501	0.563
Poly(G)			0	0	0	0
Poly(A)			0	0	0	0
Yeast RNA			0.263	0.387	0.100	0.127
Poly(A) + poly(G)			0.028	0.020	0.041	0.020
+ poly(U)						
Poly(U) + poly(A)			0.058	0.038	0.080	0.035
Poly(U) + poly(G)			0.154	0.146	0.175	0.098

Table 4. *Kinetic studies of human seminal ribonucleases*

RNAase (5 μ g/10 μ l) was used to hydrolyse synthetic poly(C) and poly(U) (0.5 mM–3 mM/100 μ l) in 0.1 M-sodium phosphate, pH 8.0 (RNAase I), 8.5 (RNAase II) and 7.5 (RNAases III and IV) at 37°C for 30 min. The RNAase activity was determined as described in the Materials and methods section. K_m and V_{max} were calculated by the Lineweaver–Burk (1934) method. Values are averages of duplicate results.

RNAase	Substrate	...	Poly(C)		Poly(U)	
			K_m (M)	V_{max} *	K_m (M)	V_{max}
I			10×10^{-9}	50	8×10^{-8}	33.3
II			2.5×10^{-9}	3.3	1.1×10^{-8}	5.3
III			8×10^{-9}	1.6	2.4×10^{-9}	1.1
IV			5.7×10^{-9}	12.6	1.5×10^{-8}	8.3

* Expressed in $\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

poly(C) over poly(U), whereas RNAase III had a higher affinity for poly(U) than for poly(C).

RNAase I was treated with 5 mM-spermine and then subjected to gel filtration on Sephadex G-75. RNAase I (approx. 90% of total protein) was dissociated into a small molecule (M_r 5000) with a considerably increased enzymic activity (5-fold over the original RNAase I activity).

Amino acid composition (as mol/100 mol and as number of residues/mol) of human seminal RNAases are given in Table 5. A similar amino-acid composition (mol/100 mol) was detected for RNAases I and IV.

Several enzyme activities were tested in these four purified seminal RNAases. No acid phosphatase (Babson & Phillips, 1966), alkaline phosphatase

(Bowers & McComb, 1975), deoxyribonuclease (Yamanaka *et al.*, 1974), phosphomonoesterase or phosphodiesterase (Burton & Peterson, 1960) or proteinase (Blackwood & Mandi, 1961) activity was detected.

Immunological characterization

Immunodiffusion and immunoelectrophoretic studies revealed an immunological identity between RNAases I and IV. Anti-(seminal RNAase I) and anti-(seminal RNAase IV) antisera showed no immunological cross-reactivity with seminal RNAase II or III or human pancreatic RNAases isolated from human pancreatic malignant ascites (M_r 30000) and normal pancreatic tissue (M_r 15000) (Lee *et al.*, 1980b), whereas a cross-reactivity was shown with other human RNAases partially purified from the lung, liver, kidney, spleen and bladder, and human serum RNAase purified by 40–75%-satd.-(NH_4)₂SO₄ precipitation and RNAase affinity chromatography. Similarly, anti-(seminal RNAase II) antiserum demonstrated an immunological reaction with seminal RNAases I and IV and other human RNAases except seminal RNAase III (Fig. 2). On the other hand, anti-(seminal RNAase III) antiserum did not exhibit any immunological reactivity with human seminal RNAase II or other human RNAases (Fig. 2), but it cross-reacted with human seminal RNAases I and IV to form a non-identical precipitin reaction.

Immunological specificity of anti-(seminal RNAase III) antiserum was further confirmed by immunohistochemical techniques. Among normal human tissues examined (lung, liver, pancreas, kidney, spleen, bladder, breast, colon, intestine, stomach, ovary, skin and prostate), only the prostate tissue section was stained by this antiserum. On the contrary, all these tissues were stainable by antisera to RNAases I, II and IV, with the exception being pancreatic tissue, which did not exhibit positive stain to anti-(RNAase I) and -(RNAase IV).

Discussion

A total of four RNAases have been isolated from human seminal plasma and characterized. Homogeneity of these purified RNAases was ascertained by conventional biochemical criteria. By monitoring protein and RNAase enzymic activity during purification, it appears that all RNAases in human seminal plasma have been isolated. These RNAases are designated as I, II, III and IV, with M_r and pI values of 78000; 16000/9.9, 133000/11.0 and 5000/4.8 respectively.

Identity of the high- M_r RNAase I is tentative. NaDodSO₄/polyacrylamide-gel electrophoresis produced one major protein band at 78000 M_r and a

Table 5. *Amino acid composition of human seminal ribonucleases*

Amino acid compositions are expressed as mol of amino acid per 100mol of enzyme ('Mol%') and number of residues per molecule of RNAase ('Res./mol.'). In order to correct for the destruction of threonine and serine or incomplete hydrolysis of valine and isoleucine during the 24h hydrolysis, the values obtained for threonine were raised 5% and those obtained for serine, valine and isoleucine were increased by 10% (Kaplan *et al.*, 1977). The contents of cysteine and tryptophan were not determined (N.D.) and were assumed to be 5%.

Amino acid	RNAase ...	Composition							
		I		II		III		IV	
		Res./mol.	Mol%	Res./mol.	Mol%	Res./mol.	Mol%	Res./mol.	Mol%
Aspartic acid		70.2	10.3	13.0	9.3	10.3	8.9	4.3	9.8
Threonine		33.8	4.9	7.7	5.5	6.7	5.7	2.1	4.9
Serine		45.5	6.6	8.4	6.0	9.1	7.9	3.0	6.0
Glutamic acid		72.0	10.5	13.6	9.7	13.4	11.5	4.4	10.0
Proline		33.3	4.9	5.5	3.9	6.5	5.6	2.1	4.9
Glycine		57.5	8.4	14.9	10.7	11.2	9.6	3.8	8.6
Alanine		66.8	9.8	15.5	11.1	9.3	7.9	4.3	9.8
Cysteine		N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Valine		47.6	6.9	9.6	6.8	8.6	7.3	3.1	7.1
Methionine		8.8	1.3	3.1	2.2	2.1	1.8	0.6	1.2
Isoleucine		20.9	3.1	6.3	4.5	4.4	3.8	1.4	3.3
Leucine		53.6	7.8	10.5	7.5	9.1	7.9	3.7	8.4
Tyrosine		20.0	2.9	3.6	2.5	2.8	2.4	1.1	2.6
Phenylalanine		27.1	3.9	4.7	3.4	3.5	2.9	1.9	4.3
Histidine		11.4	1.7	2.7	1.9	3.0	2.6	0.7	1.5
Lysine		42.2	6.2	7.9	5.6	5.9	5.1	2.7	5.9
Arginine		38.7	5.7	5.8	4.1	4.7	4.0	2.6	5.9
Tryptophan		N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total residues		649.4	94.7	132.8	94.7	110.6	94.9	41.8	95.0

Fig. 2. *Immunoelectrophoretic study of RNAases with anti-(seminal RNAase III) antiserum (a troughs) and anti-(seminal RNAase II) (b troughs)*

Seminal RNAase III (6 μ g; 5 units) and seminal RNAase II (10 μ g; 13 units) were placed in the top well as indicated. Others (partially purified RNAases) were: well 1, lung RNAases (25 μ g; 15 units); well 2, liver RNAases (30 μ g; 15 units); well 3, spleen RNAases (40 μ g; 18 units); well 4, kidney RNAases (25 μ g; 12 units); well 5, bladder RNAases (20 μ g; 12 units). The plate was washed and stained with Coomassie Brilliant Blue G-250 after immunoelectrophoresis.

minor one at 5000 M_r . Further, RNAase I was dissociated, in the presence of 5 mM-spermine, to form a small molecule of 5000 M_r , which was similar

to that of RNAase IV, with a considerable increase in enzyme activity. Additionally, amino acid analysis revealed a similar molar percentage amino acid

content for both RNAase I and RNAase IV (Table 5). In immunodiffusion studies, RNAases I and IV also revealed an identical immunoprecipitin reaction with anti-(seminal RNAase IV) or -(RNAase I) antiserum. These results suggest that RNAase I is probably an 'aggregate' of RNAase IV, as spermine has been reported to be capable of affecting aggregation-disaggregation of RNAase and simultaneously enhancing RNAase enzyme activity (Schmukler *et al.*, 1975). Certainly this notion still does not rule out the possibility that RNAase I is an 'aggregate' molecule consisting of a 5000- M_r subunit that is different from RNAase IV, although a similar amino acid composition for both RNAases I and IV would make this assumption unlikely. Alternatively RNAase IV may be a dissociated product of RNAase I that is formed during the isolation process. At present, these suggestions cannot be resolved without additional experiments.

Although RNAases II and III do not show any significant difference in M_r , each possesses distinct biological properties (Tables 1–5). Immunological studies also show that RNAases II and III are two different molecules. Additionally, biochemical and physicochemical characterization of these two seminal RNAases also differentiates them from other commonly known RNAases of human origin, such as serum RNAase (Akagi *et al.*, 1978), pancreatic RNAase (Weickmann & Glitz, 1982), leucocytic RNAase (Akagi *et al.*, 1978) and prostate RNAase-p-2 (Kuciel & Ostrowski, 1975).

The immunological properties of these four human seminal RNAases have been studied by immunoprecipitation and immunohistochemical methods. As indicated above, both RNAase I and RNAase IV possess identical antigenic determinants. Antisera to both RNAases I and IV also show immunological reaction with other human tissue RNAases (lung, liver, kidney, spleen and bladder), but fail to react with human pancreatic RNAases. Immunological studies also reveal that seminal RNAase II is antigenically similar to normal pancreatic RNAase (M_r 15000) and other human RNAases including human serum RNAases.

Of special interest from our immunological characterization is the observation on the restricted specificity of anti-(RNAase III) antiserum as revealed by immunoprecipitation techniques. Immunohistochemical study also demonstrates that RNAase III is expressed only by human prostate. An immunodiffusion study showed that anti-(RNAase III) antiserum reacted with RNAase IV, forming a line of non-identity, although anti-(RNAase IV) antiserum reacted with RNAases of various tissues. The reason for this intriguing observation is not clear at the present time. However, it is possible that, upon exposure to

RNAase IV, a conformational change in anti-(RNAase III) antibody is induced (Atassi, 1975), availing itself to bind to RNAase IV, thus forming a precipitation line in immunodiffusion. Additionally, such an antibody-induced conformational change cannot be observed in immunohistochemical studies, where the tissues have been fixed with formaldehyde (Taylor, 1978).

In conclusion, four RNAases have been isolated from human seminal plasma and characterized biochemically and immunologically. Biochemically they are apparently different from RNAases of human origin previously reported. Results also show that seminal RNAase I may be an aggregated molecule of RNAase IV. Seminal RNAases II and IV are immunochemically similar to serum RNAases, whereas seminal RNAase III appears to be a prostate-specific enzyme.

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