RIBONUCLEASE ACTIVITY ASSOCIATED WITH CHICK EMBRYO CHORIOALLANTOIC PLASMA MEMBRANES

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Ribonuclease activity was found associated with plasma membranes isolated from chick embryo chorioallantoic cells. The enzyme was solubilized with buffered 1% Triton X-100 and purified 40-fold by 1-butanol extraction and gel-filtration on Sephadex G-100. The purified RNase was found to be free of deoxyribonuclease, alkaline phosphatase and decyclizing 2',3'-phosphodiesterase activities. The enzyme is capable to degrade RNA and polycytidylic acid into acid-soluble oligonucleotides terminated in 3'-phosphate. No nucleoside monophosphates and 2',3'-cyclic nucleoside monophosphates were detected. The optimal pH of RNA and poly C hydrolysis were 7·2 and 7·8, the optimal temperatures 60 and 45°C, respectively. The purified enzyme is thermolabile, and it requires monovalent cations for the full enzyme activity.

Ribonuclease activities associated with plasma membranes have been reported in rat liver[1,2] Krebs mouse ascites tumour cells[3], chick embryo cells[4], on the surface of human cells HeLa and FL amnion cells and mouse L cells[5]. Purified Orthomyxo- and Paramyxoviruses, propagated in chick embryo chorioallantoic cells, also possessed a high RNase activity[6]. Some morphological and chemical properties of plasma membranes, isolated from chick embryo chorioallantoic cells, were described[7]. Chick embryo chorioallantoic plasma membranes possessed high activities of several associated enzymes, and RNase consistently showed a high increase of its specific activity (about 2·5-fold) over the crude cell homogenate. The aim of this work was the purification and characterization of this enzyme.

EXPERIMENTAL

Solubilization of chick embryo chorioallantoic plasma membranes. Isolation of the chick embryo chorioallantoic plasma membranes was carried out as described previously[7]. The frozen plasma membrane suspension (2 mg of membrane protein/ml of 1 mM-Tris-HCl buffer, pH 7·4) was thawed at room temperature and a buffered Triton X-100 (10% stock solution in 10 mM-Tris-HCl buffer, pH 7·2) was added to a final concentration of 1%. The suspension was gently shaken at 0°C for 60 min, kept at 4°C overnight, and centrifuged for 3 h at 105 000 *g* (Spinco L2 50B centrifuge, Ti50 angle rotor). The supernatant was used in further experiments as the source of the enzyme. About 30—50% of membrane proteins and 80—90% of original RNase activity were solubilized by treatment of the chick embryo chorioallantoic plasma membranes with buffered 1% Triton X-100.

Extraction of RNase was carried out with 1-butanol at the final concentration of 20% and subsequently of 50% (v/v) for 60 min at 0°C. After centrifugation the aqueous phase, containing the RNase activity, was dialysed for 24 h at 4°C against 1 mM-Tris-HCl buffer, pH 7·2. The enzyme specific activity increased 10—18-fold over that of chick embryo chorioallantoic plasma membranes. Extraction with 1-butanol was important for the further purification since all attemps to purity the enzyme directly from the material solubilized with buffered 1% Triton X-100 failed. At this stage of purification the enzyme became instable. However, it could be stabilized by the addition of bovine serum albumin (final concentration of 0·2%). Addition of the sucrose (5%) or glycerol (0·5%) had no protecting effect.

Column chromatography of RNase. The column of Sephadex G-100 1·5 × 33 cm was equilibrated with 100 mM-Tris-HCl buffer, pH 7·2, containing 0·2% bovine serum albumin. The chick embryo chorioallantoic plasma membrane suspension was solubilized with buffered 1% Triton X-100 and the obtained material treated with 20% and subsequently 50% 1-butanol. Water phase was concentrated on Amicon filtre PM 10 under pressure 0·5 bar/psig and 0·5 ml (650 μg) of protein fraction was layered on Sephadex G-100 column for the further purification. For the elution 100 mM-Tris-HCl buffer, pH 7·2, containing 0·2% bovine serum albumin was used. Fractions of 1·5 ml were collected and RNase activity was determined. The amount of protein per fraction was estimated in the material which was chromatographed on the same bed volume column, except that for equilibration and elution 100 mM-Tris-HCl buffer, pH 7·2 was used. Simultaneously, the RNase activity per fraction was determined.

Assay of ribonuclease activity. The degradation of high-molecular weight RNA isolated from Ehrlich ascites tumour cells and synthetic polynucleotides (polyadenylic, polycytidylic and polyuridylic acids) was followed by the formation of acid-soluble products. The incubation mixture contained 50—100 μg of the substrate, 100—200 μg of plasma membrane proteins or 1—2 μg of the purified enzyme protein in the presence of 50 mM-Tris-HCl buffer, pH 7·2 or pH 7·8 using poly C in the total volume 0·2 ml. After 45 min of incubation at 60°C with RNA (and at 45°C with synthetic polynucleotides) the reaction was stopped by the addition of 500 μg of bovine serum albumin and 0·25 ml of 6% perchloric acid. The mixture was allowed to precipitate (0°C, 20 min) and the precipitate was removed by centrifugation. With poly U ethanol was used for the precipitation instead of perchloric acid. To the reaction mixture sodium acetate was added to 1% final concentration and the nonhydrolysed poly U was precipitated by 3 volumes of 66% ethanol. Precipitation was allowed to proceed 2 h at —20°C. The absorbancy of the supernatant fraction was read at 260 nm (at 270 nm for poly C) against a blank, which contained all components except the substrate.

Alkaline phosphatase was determined according to Sinsheimer and Koerner[8]. Decyclizing 2′,3′-phosphodiesterase and alkaline phosphodiesterase (phosphodiesterase I) activities were assayed as described by Pristašová[7]. Deoxyribonuclease activity was assayed by measurement at 260 nm the acid-soluble material liberated from DNA. The reaction mixture (0·2 ml) contained 50 μg of native or denaturated (10 min at 100°C in presence of 50 mM of Tris-HCl buffer, pH 7·2) calf thymus DNA, 1—2 μg of purified enzyme protein and 1 mM-$MgCl_2$. The incubation was carried out for 2 h at 37°C. In some cases 50 μg of calf thymus DNA was incubated 45 min at 60°C with 100—200 μg of plasma membrane proteins in 50 mM of Tris-HCl buffer, pH 7·2.

Identification of the digestion products. The reaction mixtures were stopped by cooling and proteins were extracted with 2 volumes of chloroform. Digestion products of RNA and poly C were analysed by chromatography on paper Whatman No 1 in the solvent system composed of 1-butanol–ethanol–water (312 : 198 : 90) for the detection of 2′,3′-cyclic nucleoside monophosphates, and in the solvent system composed of ammonium sulfate saturated with water–

–1M ammonium acetate–isopropanol (79 : 19 : 2) for the detection of nucleoside monophosphates. The spots of digestion products located on chromatograms by means of ultraviolet light were cut out and eluted with 1 mM-Tris-HCl buffer pH 7·2. The eluates were hydrolysed with 0·3M-KOH at 37°C for 18 h and neutralised with 1M-$HClO_4$. For the detection of nucleoside 5'-monophosphates 5'-nucleotidase of *Crotalus crotalus* was used. The reaction mixture (0·5 ml) contained 0·2 ml of the hydrolysate, 20 mM-Tris-HCl buffer, pH 7·5, 20 mM-$MgCl_2$, and 25 μg of the 5'-nucleotidase. After 30 min incubation at 37°C the reaction was stopped by cooling and inorganic phosphorus was measured.

Inorganic phosphorus (P_i) was measured according to the method of Fiske and Subbarow[9]. Proteins were assayed according to the method of Lowry and coworkers[10] with bovine serum albumin as standard. RNA from Ehrlich ascites tumour cells was prepared as described previously[6]. Bovine serum albumin (fraction V, B grade), calf thymus DNA, bis-*p*-nitrophenyl phosphate, potassium salts of poly A, poly C and poly U (all A grade) were products of Calbiochem, U.S.A. Triton X-100 was obtained from Koch Light, England.

RESULTS

Characterization of the Nucleolytic Activities Associated with Chick Embryo Chorioallantoic Plasma Membranes

Chick embryo chorioallantoic plasma membranes possessed nucleolytic enzymes with high activities degrading RNA, some synthetic polynucleotides and DNA. RNA and poly C were degraded at the same rate, poly A and poly U were degraded to 30% and DNA to 50%.

The degradation of RNA by chick embryo chorioallantoic plasma membranes showed a two-component curve (Fig. 1). No more that 15% RNA was hydrolysed to acid-soluble fraction at temperature up to 40°C. At higher temperature the degradation of RNA proceeded faster and the amount of acid-soluble oligonucleotides reached the maximum at 60°C. When synthetic homopolymers were used as the substrate the amount of acid-soluble material also increased with higher temperature, but the optimum was reached at 45°C. The reaction was stimulated by monovalent cations (NH_4^+, K^+, Na^+) at the optimal concentration of 60 mmol . l^{-1}. Chick embryo chorioallantoic plasma membranes associated RNase was thermostable. During 45 min of incubation at 60°C it exerted a decrease of 18 – 20% of the original activity. The activity of chick embryo chorioallantoic plasma membranes associated RNase remained unchanged for several months when membranes were stored at –20°C. However, RNase activity rapidly decreased after several cycles of freezing and thawing.

Purification of RNase *Associated with Chick Embryo Chorioallantoic Plasma Membranes*

In the material extracted with 1-butanol several enzyme activities were been found: RNase, alkaline phosphodiesterase and phosphatase as well as decyclizing 2',3'-phosphodiesterase

(against adenosine and cytidine 2',3'-cyclic nucleoside monophosphates). Purification of RNase by ion-exchange chromatography on CM cellulose, DEAE cellulose, DEAE Sephadex as well as it adsorption on hydroxylapatite failed to separate the RNase activity from contaminating proteins. Moreover, the recovery of the enzyme activity was very low (about 10%) and after rechromatography it was completely lost. Neither purification by sucrose density gradient centrifugation[2] was effective. The contaminating proteins were distributed uniformly throughout the gradient and RNase activity sedimented in a broad peak in the top part of the gradient.

Separation of RNase activity was achieved by gel filtration on Sephadex G-100. Because of a very high lability, the enzyme was chromatographed in the presence of bovine serum albumin. In a typical experiment 500 – 700 μg of the membrane proteins were loaded on to the Sephadex G-100 column (1·5 × 33 cm) equilibrated with 100 mM-Tris-HCl buffer, pH 7·2, containing 0·2% bovine serum albumin

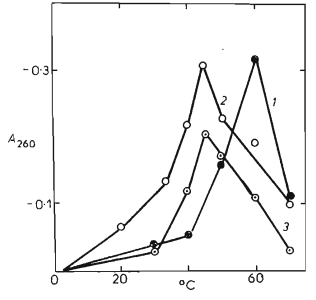

FIG. 1

Effect of Increasing Temperature on the Degradation of RNA, poly A and poly C by Chick Embryo Chorioallantoic Plasma Membrane Associated RNase

1 RNA, *2* poly C and *3* poly A. 100 μg of chick embryo chorioallantoic plasma membrane proteins (reaction with RNA and poly C) and 200 μg (reaction with poly A) were incubated with 40 μg of individual substrates for 45 min. °C, Temperature of incubation, A_{260} (or A_{270} for poly C) indicates the amount of substrates converted into acid-soluble fraction.

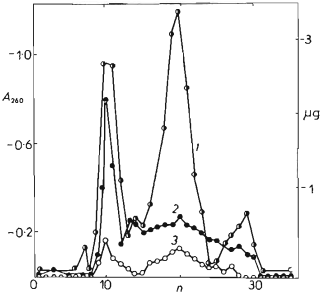

FIG. 2

Sephadex G-100 Chromatography of Chick Embryo Chorioallantoic Plasma Membrane Associated RNase

1 RNase activity A_{260}/0·2 ml, chromatography in the presence of 0·2% bovine serum albumin, 2 RNase activity A_{260}/1·5 ml, chromatography without 0·2% bovine serum albumin, *3* amount of protein μg/1·5 ml.

(Fig. 2). RNase activity eluted in three peaks and 30% of enzyme activity was recovered after the chromatography.

The first peak (fractions 9–13) containing 25–28% of the recovered RNase activity was contaminated with other enzymes. In the second peak (fractions 16–24) 60–65% of the RNase activity was eluted. The top fractions showed 35–40-fold increase of the specific activity over that of the enzyme associated with chick embryo chorioallantoic plasma membranes. In the third peak (fractions 25–31) 10–11% of RNase activity was eluted. The purification of RNase is summarized in Table I.

The separation of contaminating enzymes from RNase is shown in Fig. 3. Alkaline phosphatase is eluted with the first peak and can be separated from RNase. The majority of alkaline phosphodiesterase activity is also present in the first peak, but in front of the RNase peak a small second peak of the alkaline phosphodiesterase activity was eluted. The main (second) peak of RNase was contaminated by about 5% of the total recovered alkaline phosphodiesterase activity. Also decyclizing 2',3'-phosphodiesterase eluted in the first peak (Fig. 4). No DNase activity was detected in fractions of the purified enzyme.

TABLE I

Purification of Ribonuclease Associated With Chick Embryo Chorioallantoic Plasma Membranes

Fraction	Total protein		RNase activity		Specific activity A_{260}/mg protein	Purification
	μg/ml	%	A_{260}/ml	%		
Membranes	1 400	100	6·00	100	4·3	1
Triton X-100 (1%)	490	35	4·86	81	9·9	2·3
1-Butanol extraction (20 and 50%)	104	8	4·40	73	42·3	9·8
Sephadex G-100						
Fraction 19[a]	5	—	0·68	—	136	32
Fraction 20	5	—	0·75	—	150	35
Fraction 21	5	—	0·60	—	120	28

[a] Fractions of the second peak with the highest RNase activity.

Characterization of Partially Purified Ribonuclease

The substrate specificity of purified RNase was tested with several high-molecular weight substrates. Assays of cleavage of RNA and synthetic polynucleotides were performed under optimal conditions for chick embryo chorioallantoic plasma membranes associated enzyme. The purified enzyme did not cleavage poly A and poly U to acid-soluble oligonucleotides. On the other hand, poly C was hydrolysed as well as RNA. The optimal temperature of reaction for poly C and RNA was 45 and 60°C, respectively. The degradation of RNA by RNase was time-dependent and linear for 60 min.

Monovalent cations were required for full enzyme activity (Fig. 5). The highest stimulation of the enzyme activity occurred with sodium ions at final concentration of 60 mM. The same effect was obtained with K^+ and NH_4^+-ions. No effect on the enzyme activity was shown at 0·01 – 2 mmol . l^- concentrations of Mg^{2+}-ions. EDTA at the concentration $1 . 10^{-5}$ mol . l^{-1} had no effect on enzyme activity, higher concentrations (3 – 30 mmol . l^{-1}) inhibited the enzyme. The pH optimum was 7·2 for hydrolysis

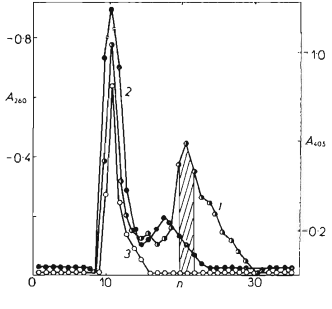

FIG. 3

Distribution of RNase, Alkaline Phosphodiesterase and Alkaline Phophatase Activities after Chromatography on Sephadex G-100

The conditions of chromatography were the same as in Fig. 2 *1* RNase (A_{260}), *2* alkaline phosphodiesterase (A_{405}), *3* alkaline phosphatase (A_{405}).

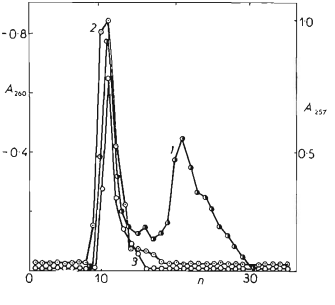

FIG. 4

Distribution of RNase and Decyclizing 2′,3′-Phosphodiesterase Activity after Chromatography on Sephadex G-100

1 RNase activity (A_{260}), decyclizing 2′,3′-phosphodiesterase activity against *2* cytidine 2′,3′-cyclic monophosphate (A_{280}) *3* against adenosine 2′,3′-cyclic monophosphate (A_{257}).

of RNA and 7·8 for the hydrolysis of poly C (Fig. 6). To follow thermal stability of the isolated enzyme, RNase preparations were exposed for 45 min to different temperatures (Fig. 7). Exposure of partially purified RNase (18-fold increase of specific activity) to 60°C exerted 55–60% lost of its activity.

When 0·1–0·5 mg of RNA and poly C were incubated with 1–2 μg of purified enzyme for 4 h at 60 and 45°C, respectively, RNA and poly C were completely degraded into acid-soluble material. The products resulting from the hydrolytic action of purified RNase on RNA and poly C were further analysed by paper chromatography. Only oligonucleotides were detected as the degradation products. After 10 to 20 min incubation of the reaction mixture no 2',3'-cyclic nucleoside monophosphates were detected. After alkaline hydrolysis of the oligonucleotides recidues and dephosphorylation of the resulting mononucleotides with 5'-nucleotidase no nucleoside 5'-monophosphates could be demonstrate. Consequently, RNase associated with chick embryo chorioallantoic plasma membranes is endonuclease which cleaves the high-molecular substrates to oligonucleotides terminated with 3'-phosphates.

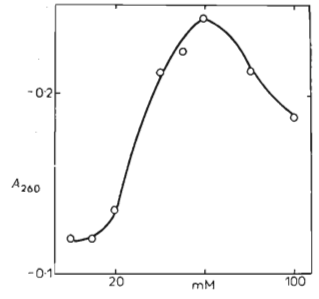

FIG. 5

Dependence of RNase Activity (A_{260}) on the Concentration of Na^+-Ions

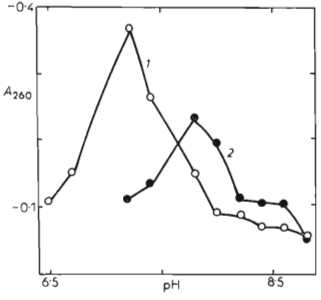

FIG. 6

Dependence of RNase Activity on pH of Reaction Mixture

The assay was done in 0·05M Na-phosphate buffer, pH 6·5 and 6·7, 0·05M-Tris-HCl buffer, pH 7·2—8·8. *1* Amount of RNA converted into acid-soluble fraction (A_{260}), *2* amount of poly C converted into acid-soluble fraction (A_{270}).

In the fractions of purified RNase (Fig. 3) small amount of alkaline phosphodiesterase was always present. Since the phosphodiesterases can remove nucleotides from the exposed ends of polynucleotide chains, the assay of RNase based on the formation of acid-soluble products should measure alkaline phosphodiesterase as well. However, we were not able to determine nucleoside monophosphates as degradation products.

DISCUSSION

Chick embryo chorioallantoic plasma membranes possess highly active RNase cleaving RNA (or poly C) into acid-soluble oligonucleotides with terminal 3'-phosphate. This observation as well as the finding that the enzyme does not hydrolyse native and denaturated DYA indicates that the RNase associated with chick embryo chorioallantoic plasma membranes may be classified as a ribonuclease-endonuclease (hydrolase)[11].

Solubilization of the enzyme was achieved in the buffered 1% Triton X-100. This detergent has been widely used in different studies of the membrane components[12]. Using buffered 0·14M-NaCl for the solubilization[1], RNase solubilized from chick embryo chorioallantoic plasma membranes amounted only 5–10% (unpublished data). However, 80–90% of the original enzyme activity was solubilized with buffered 1% Triton X-100. The necessity of the detergent for solubilization of RNase indicates that the enzyme belongs to the category of proteins equivalent to intrinsic membrane proteins[13,14]. The properties of purified RNase are similar to those of the enzyme associated with purified Orthomyxo- and Paramyxoviruses[6]. This similarity suggests that virus associated activity can be derived from the chick embryo chorioallantoic plasma membranes, which are the host-cells for these groups of RNA-containing viruses.

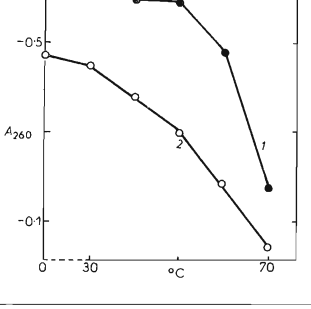

FIG. 7

Thermal Inactivation of Purified and Chick Embryo Chorioallantoic Plasma Membrane Associated RNase

Splitting rate of RNA (A_{260}) after 45 min preincubation of the enzyme at different temperatures. *1* Chick embryo chorioallantoic membrane associated RNase, *2* 18-fold purified RNase.

Chick embryo chorioallantoic plasma membranes associated RNase differs from RNase isolated from plasma membranes of Krebs mouse ascites cells[3] by its high thermolability. It markedly differs also from the RNase solubilized from rat liver plasma membranes[2]. The latter cleaves high molecular weight substrates as the exonuclease and requires Mg^{2+}-ions for its maximum activity.

The question, whether the ribonucleases detected in the plasma membranes of different vertebrate cells are constituents of these membranes themselves or contaminants will require further studies. Yannarell and Aronson[15] suggested that RNase-endonuclease, in addition to the marker-enzyme phosphodiesterase I, is localized on the rat liver plasma membranes. Our results also suggest that RNase associates with chick embryo chorioallantoic plasma membranes may be a plasma membrane enzyme.

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