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HUMAN MILK RIBONUCLEASE

B.K. DALALY *, R.R. EITENMILLER **, B.A. FRIEND and K.M. SHAHANI

Department of Food Science and Technology, University of Nebraska, Lincoln, NE 68583 (U.S.A.)

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Summary

Two components having ribonuclease (EC 3.1.27.5) activity were isolated from human milk. Each component of human milk ribonuclease (RNAase) moved at a slightly different rate when electrophoresed on polyacrylamide gel but at the same rate when ultracentrifuged. The major component had a molecular weight of approx. 14 000, an isoelectric point of pH 7.9, and exhibited a broad absorbance maximum between 277 and 281 nm. Human milk RNAase hydrolyzed yeast RNA, poly(cytidylic acid) and poly(uridylic acid) but not DNA, poly(adenylic acid) or poly(guanylic acid). Maximum activity occurred at pH 7.7 and 60°C. Amino acid analysis of the major component revealed a large number of alanine, valine, glycine and aspartic acids but no tryptophan or free sulfhydryl groups. Lysine was the N-terminal amino acid. Tryptic hydrolysis yielded 18 peptides, some of which are similar to those from bovine pancreatic RNAase. Human milk RNAase activity was increased in the presence of NaCl, KCl and sodium citrate and decreased by $CaCl_2$, $MgCl_2$, $FeSO_4$, $ZnSO_4$ and $CuSO_4$.

Introduction

Since its discovery in 1912, ribonuclease (EC 3.1.27.5) (RNAase) has been the subject of numerous investigations. Due to its high stability, low molecular weight and the ease with which it can be isolated, RNAase constitutes a model

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* College of Agriculture, University of Mosul, Haman Ilil, Mosul, Iraq.
** Department of Food Science, University of Georgia, Athens, GA 30602, U.S.A.

enzyme for study of structure and activity and is one of the most widely studied enzymes.

RNAases from human liver [1,2], pancreas [2], plasma [2,3], saliva [4,5], serum [5—7], spinal fluid [5], spleen [2,8,9] and urine [5,8] have been isolated and characterized. Bovine milk RNAase was isolated by Bingham and Zittle [10] and crystallized by Groves [11]. Chandan et al. [12] showed that milk from different mammals including human milk varied in RNAase content.

Human milk ribonuclease was found to be present as a minor contaminant in human lysozyme preparations and was successfully separated through modification of the lysozyme isolation procedure [13]. The isolated human milk RNAase contained two components with RNAase activity. The minor component with only 10% of the total activity possessed a carbohydrate moiety, while the major component with 80—90% of the total activity did not contain carbohydrate.

The objective of the present study was to determine the homogeneity of the two components of human milk ribonuclease and to further characterize the major component in order to elucidate the physiological role of ribonuclease in human milk.

Materials and Methods

The human milk samples were either collected from local hospitals or were received from the Wilmington Mothers' Milk Bank. Commercial 5X crystallized bovine-pancreatic RNAase (Nutritional Biochemicals Corp.) was the standard for the enzyme assay and yeast ribonucleic acid (Nutritional Biochemical Corp.) was the substrate. The polynucleotides, calf thymus DNA, dansyl chloride, standard amino acids and trypsin were obtained from Sigma Chemical.

Human milk RNAase isolation. Human milk contains 3—4 μg RNAase/ml. The RNAase was isolated and purified 1063-fold on a protein basis as previously reported [13]. In brief, the method involved defatting the milk by centrifugation, followed by batch adsorption of the resulting skimmed milk on Amberlite CG-50 (Na^+) at pH 6.3. After stirring in the cold for 4 h, the resin was collected in a column and washed with distilled water until the eluate was clear. Next, the RNAase was eluted with 0.2 M sodium phosphate buffer, pH 6.3. Further purification involved $(NH_4)_2SO_4$ fractionation and gel filtration on Sephadex G-50, using 0.02 M ammonium formate buffer, pH 6.3, as the eluant.

Throughout the isolation procedure, the protein content of the various fractions was determined by the method of Lowry et al. [14].

Ribonuclease activity. The activity of the RNAase was determined by a modification of the procedure of Klee and Richards [15]. The reaction mixture contained 1 ml of 1% yeast RNA and 1 ml of the enzyme. After incubation at 37°C for 30 min, the reaction was stopped by placing the test tubes in ice water for 10 min, followed by the addition of 3 ml of acidic alcohol (80 ml of 95% ethyl alcohol; 8 ml conc. HCl; 12 ml distilled water); and filtering through Whatman No. 42 filter paper. 1 ml of the filtrate was diluted to 50 ml with distilled water, and the absorbance of this solution was read at 260 nm against the blank.

Ultracentrifugal studies. Ultracentrifugal studies were carried out in a Spinco, Model E analytical ultracentrifuge using the techniques of Schachman [16]. The solvent was 0.1 M Tris-maleate buffer (pH 8.5). A pycnometer was used to determine the density of the solvent and enzyme solutions at 20°C and the apparent partial-specific volume of the RNAase, $\bar{v}_{app}$ was then calculated to be 0.724 mg/ml. The relative viscosity of the solvent to water was determined in an Ostwald viscometer.

The sedimentation coefficient $s_{20,w}$ was calculated at two enzyme concentrations and then extrapolated to infinite dilution. The apparent diffusion coefficient D_{app} was estimated from the ratio of the area under the gradient curve to the maximum height of various time periods. The values for $s_{20,w}$ and D_{app} were then substituted into the Svedberg equation to determine molecular weight.

Electrophoresis and isoelectric focusing. Polyacrylamide gel electrophoresis of both components was run in Tris-glycine buffer (pH 8.3) according to the method of Tombs et al. [17]. The major component was also electrophoresed at pH 4.0, using the system described by McAllister et al. [18].

In addition, the isoelectric point of the major component was determined in LKB Ampholine, pH 7.0—10.0, by microisoelectric focusing as described by Catsimopoolas [19]. The protein concentration was 0.1—0.2 mg per gel column.

Amino acid analysis. The amino acid composition of the major component of human milk RNAase was determined by the micro-gas-liquid chromatography method of Gehrke and associates [20—23]. The sample was hydrolyzed in 6 N HCl for 26 h at 110 ± 2°C in a closed tube under nitrogen and the *n*-trifluoroacetyl-*n*-butyl derivatives were chromatographed on a dual-column system consisting of a 1.5 × 4 mm (inner diameter) column containing 0.65% (w/w) ethylene glycol adipate (EGA) coated on dried, acid-washed 80/100 mesh Chromosorb W and a 1.0 × 4 mm (inner diameter) column with 1.5% (w/w) OV-22 coated on 80/100 mesh, high-performance Chromosorb G. 17 protein amino acids are separated on the former column, while histidine, arginine, tryptophan and cystine can be quantitatively analyzed on the latter [21]. A Varian Aerograph Model 2100 gas chromatograph equipped with a Varian Model 20 recorder and Infotronics, Model CRS 104 digital readout integrator was used. The initial temperature was 80°C and was increased at a rate of 4°C/min to 220°C.

Independent determinations of tryptophan [24] and free SH-groups [25] were also made.

N-Terminal amino acid determination. The N-terminal amino acid in the major component was determined with dansyl chloride as described by Gray [26]. The dansyl derivative was identified using TLC on Silica Gel G. The plates were developed with a chloroform/ethanol/acetic acid (38 : 4 : 3) solvent system followed by a benzene/pyridine/acetic acid (16 : 4 : 1) system as described by Deyl and Rosmus [27].

Peptide mapping. The *S*-carboxymethyl derivatives of the major component of human milk RNAase and bovine pancreatic RNAase were prepared by the method of Sela et al. [28] and then lyophilized. The lyophilized carboxymethylated proteins were dissolved in a minimum amount of water and the

solution adjusted to pH 8.5 with 0.1 N NH_4OH. A total of 3% (w/w) trypsin previously treated by the method of Bernard et al. [29] to remove chymotrypsin activity was used for proteolysis. One-half vol. of trypsin (1.5% by weight of the carboxymethylated proteins) was added, the pH adjusted to 8.5 with 0.1 N NH_4OH, and the mixture incubated at 25°C. The pH was periodically adjusted to 8.5 with 0.1 N NH_4OH. After 12 h the remaining trypsin was added and the hydrolysis continued for an additional 12 h.

The peptides were separated by TLC on cellulose according to Burns and Turner [30]. The digestion mixture was applied and then the plates were subjected to high voltage (900 V) electrophoresis at pH 2.0 for 15 min. The peptides were then separated in the second dimension with a *n*-butanol/acetic acid/pyridine/water (15 : 3 : 12 : 12) solvent system. The plates were dried and then rechromatographed in order to improve resolution. The peptides were visualized by spraying with ninhydrin-aerosol and allowing color development overnight.

Results

Homogeneity of human milk RNAase. The homogeneity of human milk RNAase was checked by ultracentrifugation and polyacrylamide electrophoresis. Fig. 1 presents a typical sedimentation pattern of the major component. The pictures were taken 1, 16, 32, 48, and 64 min after the ultracentrifuge had reached the top speed of 50 740 rev./min. When the two components of the enzyme were ultracentrifuged together, a single peak was observed.

A typical polyarylamide gel electrophoresis pattern at pH 8.3 is presented in Fig. 2. Upon electrophoresis, the major component moved slightly faster than the minor component. Electrophoresis of the major component at pH 4.0, revealed a single band.

Molecular weight. The $s_{20,w}$ of the major component was 1.96 S. The D_{app} at 20°C in 0.1 M Tris-maleate buffer (pH 8.5) was $12.09 \cdot 10^{-7}$ cm^2/s. The molecular weight of the major component of human milk RNAase was found to be 14 024 using the Svedberg equation.

Isoelectric point. Isoelectric focusing of the major component resulted in a

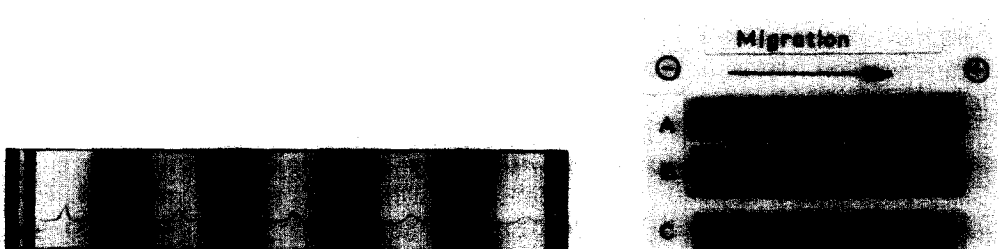

Fig. 1. Ultracentrifuge sedimentation velocity patterns of the major component of human milk RNAase at 20°C in a synthetic boundary cell. The pictures were taken 1, 16, 32, 48 and 64 min after attaining a speed of 50 740 rev./min. Protein concentration was 0.4%.

Fig. 2. Polyacrylamide gel electrophoresis patterns at pH 8.3. Gel A contained the minor and major components electrophoresed together. Gel B is the minor component and gel C is the major component. The RNAase concentration was 400 μg/ml and sample size was 40 μl.

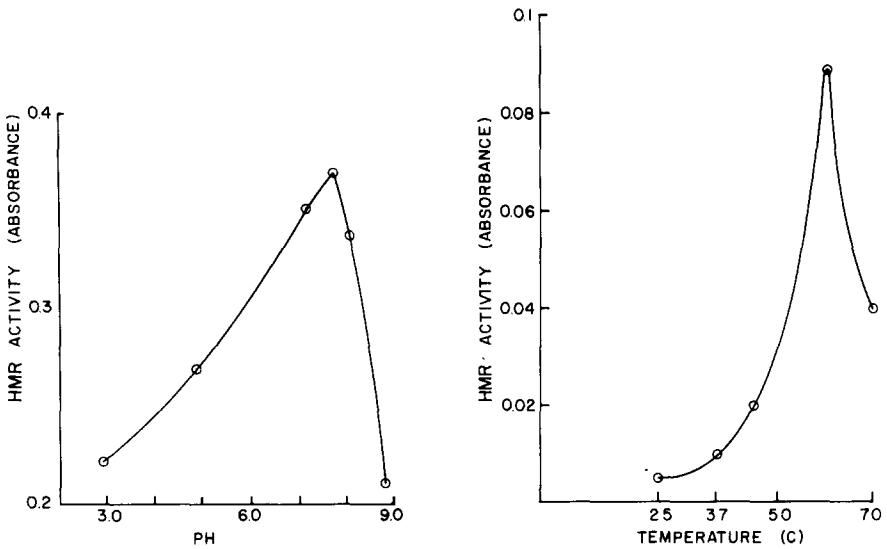

Fig. 3. Effect of pH and temperature on activity of human milk RNAase. The pH optimum at 37°C was determined in 0.2 M acetate buffer (pH 3.6—5.6), 0.2 M phosphate buffer (pH 6.0—8.0), 0.2 M borate buffer (pH 4.0—10.0), 0.2 M Tris-HCl buffer (pH 6.7—9.0) and 0.2 M glycine buffer (pH 8.6—10.0) and the curve represents an average of these determinations. The thermal inactivation was determined by incubating RNAase for 30 min in 0.2 M borate buffer (pH 7.8). HMR, human milk ribonuclease.

single band at pH 7.9 for each of three separate determinations.

Ultraviolet absorption spectrum. The ultraviolet absorption spectra of human milk RNAase and bovine pancreatic RNAase were determined for comparative purposes. As presented in Fig. 3, some differences in the ultraviolet absorption spectra of the two ribonuclease proteins are apparent. The major component exhibited a broad maximum absorbance from 277 to 281 nm, while bovine pancreatic ribonuclease exhibited a narrow maximum absorbance at 277 nm.

pH Optimum. The activity of the major component at 37°C was studied over the pH range 3.0—10.0 and the results of this study are presented in Fig. 4. Maximum activity was obtained at pH 7.7, in 0.2 M borate buffer. Similar studies with the minor component showed the same pH optimum.

Thermal inactivation. The activity of the major component was determined over a range of 25—70°C and, as shown in Fig. 4, had maximum activity at 60°C. The activity of the minor component, either alone or in conjuction with the major component, showed the same activity with respect to temperature. However, in order to elucidate the behavior of the human milk RNAase under physiological conditions, all other activity tests were carried out at 37°C.

Specificity. Poly(adenylic acid), poly(cytidylic acid) and poly(uridylic acid) in potassium form and poly(guanylic acid) in sodium form were used as substrates in this study. The substrates were dissolved in 0.1 M borate buffer (pH 7.7) and activity was assayed at 37°C. As shown in Table I, the major component hydrolyzed all the pyrimidine substrates at the same rate, while the purine substrates were not hydrolyzed by the enzyme. Similarly, the human milk RNAase did not hydrolyze DNA.

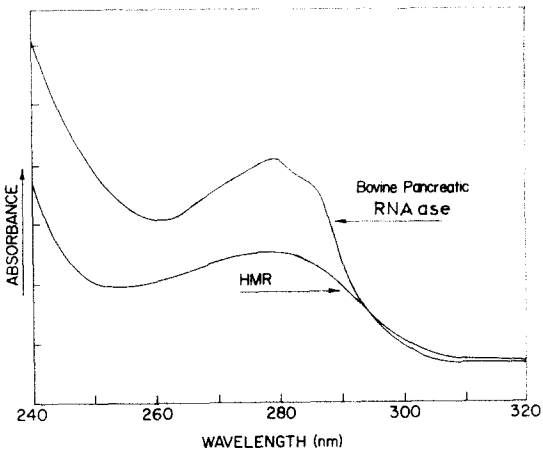

Fig. 4. Ultraviolet spectra of human milk RNAase and bovine pancreatic RNAase. The enzymes were prepared in 0.02 M formate buffer (pH 6.2) at a concentration of 550 μg of RNAase. HMR, human milk ribonuclease.

Amino acid analysis. Table II presents the amino acid composition of the major component of human milk RNAase. The number of amino acid residues is based on 14 000 molecular weight, determined by the sedimentation method. For comparison, the composition of bovine pancreatic RNAase analyzed by the same method [20,23], and the composition based on sequencing data [31], are also given. The major component contains more alanine, valine, glycine and leucine, and less tyrosine, lysine, and cystine. Tryptophan was absent in both enzymes as determined by GLC and titration. Similarly, there were no free sulfhydryl groups in the main component as determined with *p*-mercuribenzoate. Therefore, all half-cystine residues appear to be involved in intramolecular disulfide bonds in both the major component (six bonds) and the bovine pancreatic RNAase (four bonds).

N-Terminal amino acid determination. The N-terminal amino acid of the major component was determined with dansyl chloride. Development in both solvent systems revealed didansyl lysine, indicating that the human milk RNAase major component contains lysine in the N-terminal position.

Peptide maps. Peptide maps of the tryptic digest of both the major component and bovine pancreatic RNAase are shown in Fig. 5. Only peptides of low intensity were circled in the pancreatic RNAase map.

TABLE I

BASE SPECIFICITY OF HUMAN MILK RIBONUCLEASE

Substrate	Absorbance at 260 nm
Blank	0.0
Yeast RNA	0.15
Poly (cytidylic acid)	0.2
Poly (uridylic acid)	0.2
Poly (adenylic acid)	0.0
Poly (guanylic acid)	0.0

TABLE II

AMINO ACID COMPOSITION OF HUMAN MILK RNAase

For human milk RNAase approx. 100 μg of the major component (carbohydrate free) were hydrolyzed in 1 ml 6 N HCl for 26 h at 110°C in a closed tube prior to derivatization. Each value represents an average of two chromatographic determinations made on the same hydrolysate. Ornithine was used as the internal standard and the number of residues based on a molecular weight of 14 000. The method for bovine pancreatic RNAase was adapted from Gehrke et al. [20] and Roach and Gehrke [23] and based on a molecular weight of 14 000.

Amino acid	Gas-liquid chromatography		Literature value * bovine pancreatic RNAase
	Human milk RNAase	Bovine pancreatic RNAase	
Alanine	18	14	12
Valine	13	9	9
Glycine	15	4	3
Isoleucine	5	2	3
Leucine	10	2	2
Proline	7	4	4
Threonine	6	10	10
Serine	7	16	15
Methionine	1	4	4
Phenylalanine	4	3	3
Aspartic acid	12	16	15
Glutamic acid	13	13	12
Tyrosine	1	6	6
Lysine	5	11	10
Arginine	5	4	4
Histidine	4	4	4
Cystine	6	4	4
Tryptophan	0	0	0
	132	126	120

* Based on sequence of pancreatic RNAase determined by Smyth et al. [31].

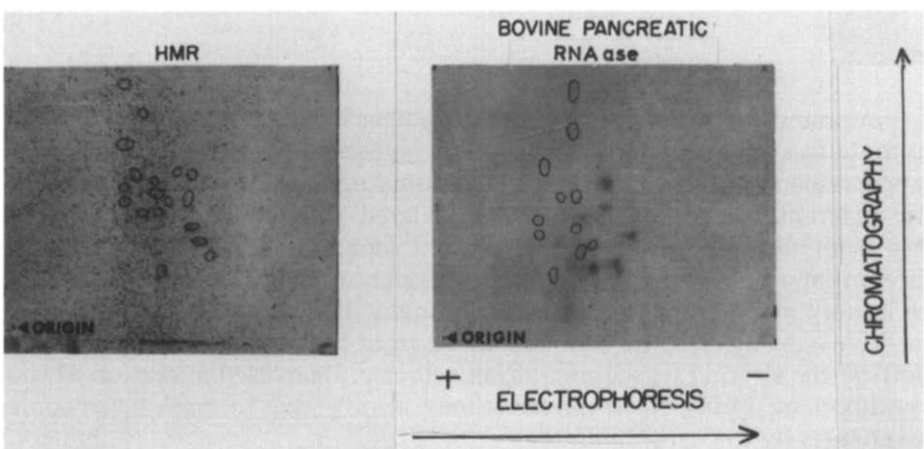

Fig. 5. Peptide maps obtained from the tryptic digests of human milk RNAase and bovine pancreatic RNAase. HMR, human milk ribonuclease.

TABLE III

EFFECT OF THE ADDITION OF SALTS ON THE ACTIVITY OF HUMAN MILK RNAase

Salt	Final concentration (mM)	Relative activity (%)
Control	—	100
NaCl	100	166
NaCl	200	255
NaCl	400	140
$NaC_6H_5O_7$	50	160
KCl	100	150
KCl	200	260
$CaCl_2$	100	45
$CaCl_2$	300	0
$MgCl_2$	100	20
$MgCl_2$	200	30
$FeSO_4$	$2 \cdot 10^{-1}$	42
$FeSO_4$	2	0
$CuSO_4$	2	15
$CuSO_4$	6	7.5
$ZnSO_4$	$2 \cdot 10^{-1}$	47
$ZnSO_4$	2	25
$MnSO_4$	12.5	14
$MnSO_4$	25	0

A total of 18 peptides could be counted in the tryptic digest of human milk RNAase. This is eight peptides in excess of the theoretical value since human milk RNAase contains a total of 10 lysine and arginine residues. Similarly, 21 peptides, or six in excess of the theoretical value, were obtained for bovine pancreatic RNAase. Both human milk and bovine pancreatic RNAase showed three fast moving spots upon chromatography.

Effect of salts. 0.1 ml of salt solution was added to 0.9 ml of enzyme solution to give the desired concentration in the final reaction mixture, and the activity determined as described earlier. As shown in Table III, the presence of Na^+ and K^+ increased activity, while the remaining cations decreased activity.

Discussion

Two compounds with RNAase activity have been isolated from human milk, a carbohydrate-free major component and a glycoprotein minor component. They are ultracentrifugally the same but chromatographically and electrophoretically different. Dixon and Webb [32] isolated two ultracentrifugally pure RNAases from rat muscle which yielded eight separate electrophoretic bands. The differential activity of the milk RNAases may be attributed to the carbohydrate moiety attached to the minor component. The small increase in molecular weight would increase its rate of elution from the Sephadex column without significantly altering its sedimentation velocity. Decreased migration of the minor component during electrophoresis may also be due to masking of some negative charges by the carbohydrate.

Reinhold et al. [33] isolated a highly heterogeneous porcine pancreatic RNAase with eight electrophoretically distinguishable components. All had

identical amino acid compositions but different carbohydrate contents. The extent of glycosidation had no effect on enzymatic activity. Similarly, Plummer [34] showed that RNAases C and D isolated from bovine pancreatic juice were identical to RNAases A and B except for carbohydrate content.

Eylar [35] suggested that the carbohydrate moiety of a glycoprotein is not associated with the enzymatic role of a protein but rather acts as a chemical label, which promotes the extracellular transport of the newly synthesized glycoprotein. Since both components of human milk RNAase have the same sedimentation rate, pH optimum and thermal stability and differ only in the presence (or absence) of a carbohydrate moiety, they are probably enzymatically the same.

The molecular weight of the major component was determined by the sedimentation velocity method. Since the amount of RNAase in human milk is small, independent diffusion studies could not be performed accurately. Instead, the diffusion coefficient was estimated as described earlier and should be regarded as approximate. The molecular weight was found to be approx. 14 000. Likewise, the molecular weight of the minor component should be slightly greater than this value because of its carbohydrate moiety.

Although the molecular weight of the human milk RNAase is quite similar to that of bovine pancreatic RNAase, the amino acid composition is quite different (Table II). Only one tyrosine residue was found in the major component, while six are present in bovine pancreatic RNAases. The ultraviolet absorption spectra also indicate more tyrosine in the pancreatic RNAase as compared to the milk RNAase. Since the difference is not 6-fold, it is likely that some tyrosine was lost during hydrolysis, prior to amino acid analysis. Since fewer residues are initially present in the human milk RNAase, the effect would be more pronounced as compared to the bovine pancreatic RNAase.

Neither of the RNAases contains tryptophan or free half-cystine residues. Both have lysine as the N-terminal group and approximately the same ratio of acidic to basic residues resulting in similar isoelectric points.

Tryptic hydrolysis of both the human milk major component and bovine pancreatic RNAase yielded more peptides than theoretically expected. Takahashi [36] also found several peptides in excess of the theoretical in T1 RNAase because of cleavage after two tyrosine residues during tryptical hydrolysis. Inagami and Sturtevant [37] have suggested that trypsin may contain an inherent chymotrypsin-like activity which could account for the excess peptides obtained in the present study.

The effect of the ionic species and ionic strength of the reaction medium on the activity of RNAase has been well documented [3,4,7,38]. Eichel et al. [4] noted that the activity of human salivary RNAase could be increased 80% by the addition of NaCl, to increase the ionic strength to 0.167. Although the activity of human plasma RNAase was decreased significantly as the buffer concentration was reduced from 0.1 M to 0.01 M, the activity against RNA could be restored by the addition of various cations to the dilute buffer mixture [3].

The activity of the major component of human milk RNAase was increased significantly by the addition of the monovalent cations Na^+ and K^+. The activity of human serum RNAase is also increased in the presence of Na^+ or K^+ [7]. However, the activity of serum RNAase is increased with Mg^{2+}, Ca^{2+} and Co^{2+}

[7] and plasma RNAase with Ca^{2+} [3], while the activity of the milk RNAase was decreased with all divalent cations tested. Both serum [7] and milk RNAase showed less activity in the presence of Fe^{2+}, Cu^{2+} and Zn^{2+}.

It is not known whether human milk RNAases are synthesized in the mammary gland or in some other organ of the body and transported to the mammary gland. In cows it is thought that pancreatic RNAase appears in milk as a result of intestinal absorption and transport. Although the milk and pancreatic RNAases have similar amino acid compositions [39] and are serologically identical [40], they each possess different carbohydrate moieties [41]. Thus, synthesis of bovine RNAase by both the pancreas and mammary gland cannot be ruled out [41].

In humans, pancreatic and serum RNAase are serologically related to each other [2] but not to human liver, human spleen or bovine pancreatic RNAase [2,9]. Their pH optimum of 8.0 [2,3] is also similar to the value of 7.7 determined for the human milk RNAase. However, the molecular weight of 33 500 for pancreatic [2] and 32 000 for plasma RNAase [3] is more than twice that of human milk RNAases. On the other hand, human liver RNAase with a species of molecular weight 14 000 [1], human spleen with a species of 13 600 [9] and human serum with a species of 13 000 [7], more closely resemble the molecular weight of 14 000 determined for the human milk RNAase. Until more information is available concerning the composition and structure of the various RNAases found in the human, the source of human milk RNAase remains highly speculative.

In nature, RNAase appears to exist together with lysozyme in several biological systems. While isolating lysozyme from a fungus belonging to the genus *Chalaropsis*, Hash and Rothlauf [42] observed that RNAase was eluted just ahead of lysozyme from the column. Also, Manwell [43] has suggested that RNAase and lysozyme may be related via an ancestral gene. However, no information is available concerning any functional relationship between lysozyme and RNAase. Their activities may be complementary since lysozyme cleaves the cell wall of the susceptible organisms and thereby exposes the protoplasmic RNA to hydrolysis by the RNAase.

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