

Purification and Characterization of a High Molecular Weight Ribonuclease from Human Milk*

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We have purified a high molecular weight ribonuclease (hmRNase) from human milk by a two-step column chromatographic procedure and characterized the enzyme. The molecular mass of hmRNase is 80 kDa as determined from SDS-polyacrylamide gel electrophoresis. The pH optimum of the enzyme is in the range of 7.5–8.0, similar to other secretory RNases. hmRNase is pyrimidine-specific and cleaves the phosphodiester bond 3' to a pyrimidine residue. It selectively degrades the pyrimidine strand in poly(rA):poly(rU) and poly(dA):poly(rU) double stranded substrates. The extent of degradation for naturally occurring RNAs vary in the order tRNA < rRNA < mRNA at low enzyme concentrations. hmRNase shows allosteric behavior with positive cooperativity in its reaction on polynucleotide substrates. The activity of the enzyme is enhanced in the presence of monoribonucleotides. Antiserum obtained against purified hmRNase did not cross-react with low molecular weight RNase which is also present in milk. In addition, an immunologically cross-reacting species could not be detected in the serum, suggesting the origin of hmRNase in the mammary gland but not blood.

Multiple RNase activities have been reported from several body fluids of mammals (1–4). The complete or partial sequence information on human RNases currently available suggests two predominant categories. The first category is the pancreatic RNase-like enzyme, which is also present in human urine and kidney (5–7). The second type is the nonsecretory RNase from urine (8) which shares NH₂-terminal sequence identity with RNase from human kidney, liver, and erythrocytes (7, 9, 10).

The ubiquitous presence of RNases in body fluids has made it difficult to assign a precise role for these enzymes. However, for RNase activity in milk, a protective role in retroviral infection has been envisaged. It was demonstrated that milk RNase inhibited the reverse transcription of the RNA genome of mouse mammary tumor virus (11). This observation is significant, because mouse mammary tumor virus is transmitted through milk from mother to progeny in several strains of mice (12, 13). It was also shown that RNA-dependent DNA synthesis by a retrovirus, avian myeloblastosis virus, incubated in human milk plasma was inversely proportional to the amount of ribonuclease present in the milk samples (14).

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These studies suggested that the decrease in RNA-dependent DNA synthesis was due to degradation of the RNA genome.

Furthermore, the levels of RNase present in milk samples from donors belonging to the Parsi community were found to be lower compared to those in milk samples from other ethnic groups (14). Parsis constitute a highly consanguineous community in Western India and have about three times higher age-adjusted incidence of mammary cancer compared to that in other Indian communities (15, 16). Jussawala *et al.* (15) have also reported that among Parsi women, breast cancer accounted for 40% of all malignancies. There was thus an indication for an inverse correlation between mammary tumor incidence and the amount of RNase activity found in the milk plasma (14). It was therefore of interest to study the RNase activity present in human milk.

Recently, RNases have been recognized to constitute a superfamily, in which several diverse proteins such as angiogenin and eosinophil cationic protein are shown to share sequence homology with ribonucleases (17–19). These proteins possess ribonuclease activity in addition to other diverse functions (18, 20–22). In addition, a neurotoxin isolated from the granules of an eosinophil named eosinophil-derived neurotoxin was shown to have ribonuclease activity (21) and has a sequence identical to human nonsecretory ribonuclease (23).

In an earlier paper the high molecular weight ribonuclease from human milk (hmRNase)¹ was shown to be an isoform of lactoferrin, an 80-kDa glycoprotein present in milk with a major role in iron storage and transport (see "Discussion") (24). In this paper, we report purification of the high molecular weight ribonuclease from human milk and its enzymatic characterization.

EXPERIMENTAL PROCEDURES

Materials—[³H]Uridine and [³H]orotic acid were obtained from Bhabha Atomic Research Centre, India. Agarose-5'-(4-aminophenylphosphoryl)uridine 2'-(3')-phosphate, poly(rG), and poly(rU) were obtained from P-L Biochemicals. RNA used for activity staining of gels was obtained from Calbiochem, phosphocellulose P11 was from Whatman. Uranyl acetate was purchased from Fisher. Polyethylenimine cellulose sheets impregnated with fluorescent dye were purchased from Fluka AG. Peroxidase-conjugated anti-rat (rabbit) antibody was purchased from Miles-Yeda. GMP and dGMP were obtained from Nutritional Biochemicals. All other fine biochemicals, including di- and polynucleotides used in the present experiments were obtained from Sigma.

Milk Samples—Milk was obtained from volunteers at local hospitals 3–10 days post partum, brought to the laboratory in an ice bath, and stored at –20 °C. The samples were thawed just before use, centrifuged at 100,000 × *g* for 1 h at 4 °C, and the pellet and the fat button were discarded. The clear middle layer of plasma was dialyzed in 100 volumes of buffer containing 0.01 M sodium phosphate (pH 6.5), 0.05 M NaCl, and 1 mM PMSF.

¹ The abbreviations used are: hmRNase, high molecular weight ribonuclease from human milk; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.

Isolation of RNA—*Escherichia coli* was cultured in the presence of 2 mCi of [³H]uridine (41 mCi/mmol) per liter, harvested in mid log phase, and labeled RNA was isolated according to the procedure described by Midgley (51). Tritiated rat liver RNA was obtained by injecting rats intraperitoneally with 2 mCi of [³H]orotic acid (41 mCi/mmol). The rats were starved for 24 h, and their livers were homogenized in 5 volumes (w/v) of buffer containing 0.05 M Tris-HCl (pH 7.5), 0.025 M KCl, 0.005 M MgCl₂, and 0.25 M sucrose. The homogenate was centrifuged at 12,000 × *g* for 15 min at 4 °C. The upper two-thirds portion of the supernatant was mixed with 1/20th volume of freshly prepared 10% (w/v) sodium deoxycholate and centrifuged at 105,000 × *g* for 90 min at 4 °C. Transfer RNA was isolated from the supernatant, and rRNA from the ribosome pellet was suspended in 25 ml of buffer containing 0.025 M sodium acetate (pH 5.1), 0.15 M NaCl, 0.005 M MgCl₂, 0.001 M EDTA, and 0.5% w/v bentonite, by the phenol extraction method described by Midgley (51). Poly(A⁺) RNA was isolated from rat liver according to the procedure of Skup *et al.* (52), and avian myeloblastosis virus RNA was isolated as described by Das and Mink (53).

Before using as a substrate for RNase, RNA preparations were routinely checked for purity and integrity by electrophoresis on a 2.5% polyacrylamide gel (23 × 10 cm) cast in the buffer, 0.036 M Tris, 0.03 M sodium phosphate (pH 7.8), and 0.001 M EDTA (Tris-phosphate buffer). Electrophoresis was carried out in the same buffer at 40 mA for 3 h, and the RNA bands were detected by staining with 0.2% (w/v) Methylene Blue.

RNase Assay Using ³H-Labeled Substrates—The standard reaction mixture contained 0.01 M Tris-HCl (pH 7.5), 0.05 M NaCl, 40 μg of ³H-labeled RNA (1000 cpm/μg RNA) and an aliquot of the enzyme in a final volume of 50 μl. Incubations were done for 15 min (or as mentioned in the individual legends to the figures) at 37 °C. An aliquot (25 μl) of reaction mixture was spotted on a 2 × 10-cm strip of Whatman No. 3 chromatography paper pre-streaked with glacial acetic acid. The strip was chromatographed in a solvent system containing 95% ethanol and 1 M ammonium acetate (1:1, v/v). The strip was dried, the origin was cut out, and the amount of undegraded RNA was determined by measuring the radioactivity present at the origin of the strip (54). One unit of RNase was defined as the enzyme needed for degradation of 1 μg of *E. coli* [³H]RNA in 15 min under the assay conditions mentioned above.

Assay of RNase Using Unlabeled Substrates—The activity of RNase on unlabeled substrate was measured by a modification of the procedure described by Kalnitsky *et al.* (55). The enzyme was incubated with 50 A₂₆₀ units of the substrate at 37 °C in 0.01 M Tris-HCl (pH 7.4) containing 0.05 M NaCl. The undegraded substrate was precipitated by incubation with an equal volume of a solution containing 0.5% (w/v) uranyl acetate and 16% (w/v) perchloric acid. The mixture was chilled in an ice bath for 30 min, centrifuged at 10,000 × *g* for 15 min. The absorbance (A₂₆₀) of the supernatant was determined for quantitating the activity. With poly(rC) and poly(rA):poly(rU) as substrates, hyperchromicity assay described by Kalnitsky *et al.* (55) was used. Double stranded substrates poly(rA):poly(rU) and poly(dA):poly(rU) were freshly annealed before use, and their double strandedness was confirmed by melting curves.

Analysis of the Degradation Products of Dinucleotides—The dinucleotides CpG or GpC were incubated with hmRNase, RNase A, or phosphodiesterase in 10 μl of 0.01 M Tris-HCl (pH 7.5) at 37 °C for 30 min. Each of the assay mixtures was spotted on a polyethylenimine cellulose sheet impregnated with a fluorescent dye, chromatographed with distilled water, and the spots were observed under UV light. The nucleosides migrate with characteristic relative mobilities, and the nucleotides remain at the origin. The sheet was dried and rechromatographed in 0.075 M Tris-HCl (pH 8.0) until the buffer front was just below the spot corresponding to guanosine and the mono- and dinucleotides move from the origin.

Protein Estimations—Protein concentration was determined according to Lowry *et al.* (56).

Gel Electrophoretic Procedures—SDS-PAGE was performed according to Laemmli (57), and the protein bands were visualized by silver staining on SDS-PAGE. Glycosylation was tested using periodic acid Schiff's reagent. Isoelectric focusing was done according to the method described by O'Farrell (58).

Staining of the gels after SDS-PAGE for the detection of RNase activity (zymogram of RNase) was carried out as follows: The gel was fixed in 25% isopropanol buffered with 10 mM Tris-HCl (pH 7.5) for 2 h at room temperature. It was then washed free of isopropanol in the above buffer and was incubated in a solution containing 2.5 mg/ml RNA at 37 °C for 12 h. The gel was washed in Tris buffer with

three changes for 30 min each. Staining for RNA was carried out using a 0.1% solution of Methylene Blue in distilled water. Continuous shaking at 75 rpm was maintained throughout the process. Appearance of clear bands in a dark blue background after destaining indicated the presence of RNase.

Amino Acid Analysis—The amino acid composition was determined after hydrolysis of hmRNase in 6 N HCl at 100 °C *in vacuo*. The cysteine content was determined after performic acid oxidation of the protein and also by a spectrophotometric method (59).

Immunological Procedures—Antisera against purified hmRNase were raised by primary and secondary immunizations (0.5 mg each) of rats. Antisera were used in Ouchterlony double diffusion analysis (60) and for immunoblotting experiments (61).

RESULTS

The zymogram of human milk plasma revealed two bands with RNase activity (Fig. 1, lane 1) corresponding to molecular masses of 80 and 14 kDa, respectively. RNase with a molecular mass as high as 80 kDa as measured under denaturing conditions has not been observed so far in any vertebrate body fluids. The 80-kDa enzyme (hmRNase) was purified to homogeneity and characterized further.

Purification of hmRNase from Human Milk Plasma—The dialyzed milk plasma (50 ml) was adsorbed onto a phosphocellulose column (1.5 × 30 cm), equilibrated with 10 mM sodium phosphate (pH 6.5), 50 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF). After washing the column with the same buffer containing 0.2 M NaCl, the adsorbed proteins were eluted with a linear gradient of 0.2–1.5 M NaCl (Fig. 2). Two peaks of RNase activity were obtained, and the fractions corresponding to the major peak eluting at 0.7–1.0 M NaCl were pooled, concentrated, and dialyzed in 10 mM Tris-HCl (pH 7.5) and 1 mM PMSF (Tris buffer). The enzyme was chromatographed on an agarose 5'-(4-aminophenylphosphoryl)uridine 2'-(3')-phosphate column equilibrated in Tris buffer. The column was washed with the same buffer, and the enzyme was eluted with Tris buffer containing 0.5 M NaCl (profile not shown). hmRNase is a major component of the proteins present in the milk plasma, and after purification

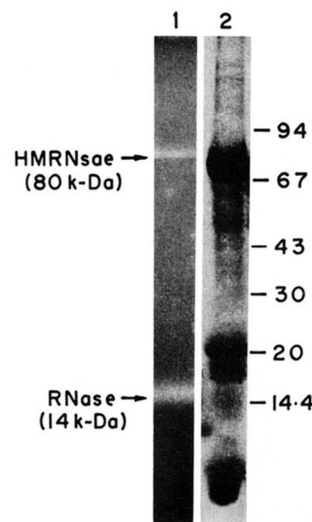


FIG. 1. Zymogram of crude milk plasma. The milk plasma was electrophoresed on a 12.5% SDS-PAGE and was washed free of SDS in a buffer containing 0.01 M Tris-HCl (pH 7.5), 0.05 M NaCl, 25% isopropanol. The gel was incubated in the above buffer containing 2.5 mg/ml RNA for 12 h at 37 °C with constant shaking and washed with Tris buffer. RNA on the gel was stained with Methylene Blue and destained in distilled water as described under "Experimental Procedures." Lane 1, zymogram; lane 2, gel stained with Coomassie Blue to detect total protein.

FIG. 2. Elution profile of hmRNase from phosphocellulose column. Milk plasma was adsorbed onto the column in 0.01 M sodium phosphate (pH 6.5), 0.05 M NaCl, and 0.001 M PMSF, washed with the same buffer containing 0.2 M NaCl. The adsorbed proteins were eluted with a gradient of 0.2–1.5 M NaCl. RNase activity (●) was assayed using *E. coli* [³H]RNA as described under "Experimental Procedures." Protein was monitored at A₂₈₀ (○).

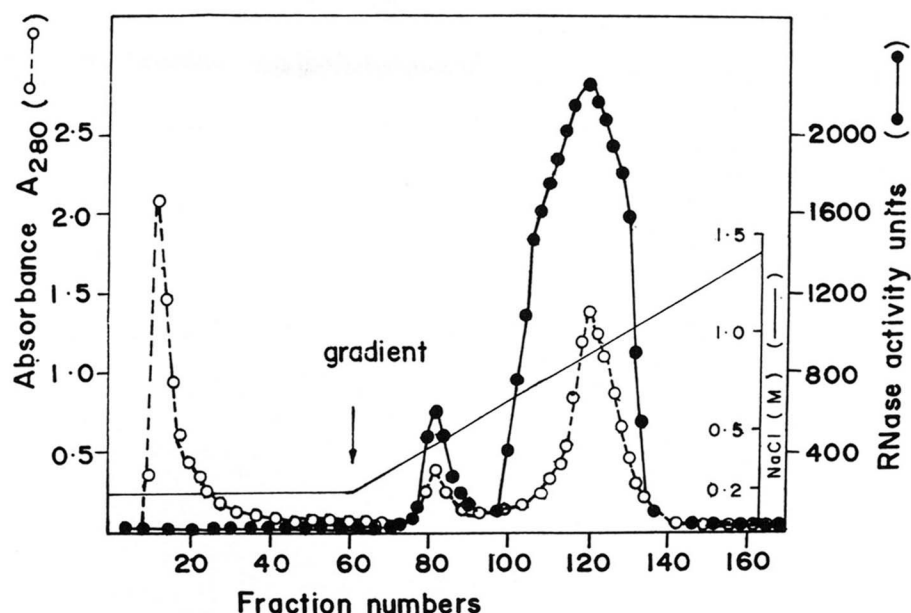


TABLE I
Purification of hmRNase

Aliquots of the milk plasma and pooled fractions containing hmRNase were assayed using *E. coli* [³H]RNA as a substrate, as described under "Experimental Procedures."

Step	Total protein mg	Total activity units	Specific activity units/mg	Yield %	Purification -fold
1. Crude milk plasma	800	21,760	27.2		
2. Phosphocellulose column	16.5	16,087	975	81	36
3. Affinity column	12.4	14,552	1,173	67	43

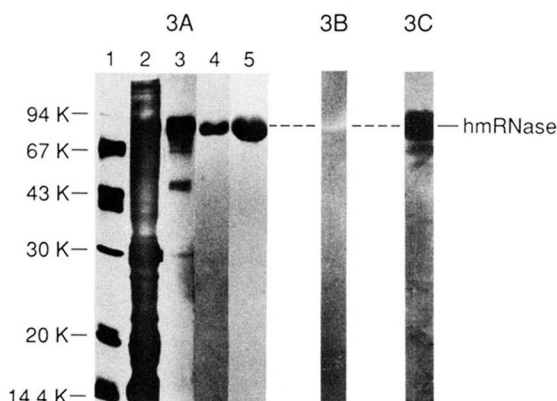


FIG. 3. SDS-PAGE profiles of hmRNase. A, SDS-PAGE profiles of samples from various stages of purification. Electrophoresis was carried out in 10% SDS-polyacrylamide gel and was stained by the silver staining. Lane 1, standard molecular weight markers; lane 2, crude milk plasma; lane 3, phosphocellulose column pooled fraction; lane 4, affinity column fraction; lane 5, affinity column fraction loaded 5-fold greater than in lane 4. B, zymogram of purified hmRNase. The zymogram was obtained under conditions identical to those described in the legend to Fig. 1. C, Western blot of the crude milk plasma. Rat antiserum raised against purified hmRNase was used for blotting.

there is a 43-fold enrichment of the enzyme activity (Table I).

The protein profiles of the crude milk plasma and each of the steps of purification are shown in Fig. 3A. The ribonuclease purified by this procedure appeared as a single band on

SDS-PAGE (Fig. 3A) or on anodic and cathodic native gels (not shown). The hmRNase preparation was free of the 14-kDa RNase as seen from the lack of low molecular weight band in the zymogram (Fig. 3B) or in Western blot of the crude milk plasma using antibodies raised against hmRNase (Fig. 3C). When checked for enzyme activities such as DNase, phosphodiesterase, alkaline phosphatase, or nonspecific esterases, no such activities could be detected in purified material (results not shown).

Molecular Weight—From the SDS-PAGE profile (Fig. 3A) the subunit molecular mass of the purified hmRNase was found to be 80 kDa. The mobility of the enzyme on SDS-PAGE was not affected by reduction with β -mercaptoethanol or by boiling (not shown). This band could also be detected by activity staining of the SDS-polyacrylamide gel (Fig. 3B). These results suggest that the enzyme is composed of a single polypeptide. The positive staining of the RNase band with periodic acid Schiff's stain indicated the glycoprotein nature of hmRNase (not shown).

Amino Acid Analysis—The amino acid composition of hmRNase obtained as described under "Experimental Procedures" is presented in Table II. Since hmRNase was proposed to be an isoform of lactoferrin, the amino acid composition of human lactoferrin and human pancreatic RNase calculated from the published sequence (25, 26) are also given for comparison. It can be seen that the experimental values for hmRNase are in good agreement with that of lactoferrin for many amino acids. However, a few differences exist, such as for tryptophan and cysteine. hmRNase reveals a rather high cysteine content of 8 mol % measured in the form of cysteic acid after oxidation of the protein by performic acid, prior to hydrolysis. In order to confirm this result, the spectrophotometric method using titration with 5,5'-dithiobis-(2-nitrobenzoic acid) was carried out for the determination of cysteine. The free sulfhydryl groups estimated with unreduced protein was very low (0.3 mol %), whereas after reduction with dithiothreitol it showed a value of about 9 mol % which is in accordance with the cysteine content estimated after performic acid oxidation.

Thermal Stability—On heating the hmRNase, 60% of the activity with *E. coli* [³H]RNA as the substrate under standard assay conditions was lost at 60 °C in 5 min, while at 90 °C,

TABLE II

Amino acid composition of hmRNase

Hydrolysis of purified hmRNase was carried out for 24 and 72 h as described under "Experimental Procedures." Data for valine, leucine and isoleucine were from analysis after 72-h digestion. The values for other amino acids were interpolated to 0 h and presented in the table. Data for human Lactoferrin (25, 26) and human Pancreatic RNase (5) were derived from published sequences.

Amino acid	Mole %		
	hmRNase	Lactoferrin	Pancreatic RNase
Aspartic acid + asparagine	9.2	5.3 + 4.5	4.7 + 7.0
Threonine	4.8	4.2	6.2
Serine	6.8	7.7	12.5
Glutamic acid + glutamine	10.5	5.6 + 4.2	4.7 + 5.5
Proline	4.4	5.8	5.5
Glycine	7.0	7.7	3.9
Alanine	8.4	8.9	3.1
Cysteine	7.6 ^a	4.6	6.2
Valine	8.2	6.9	7.8
Methionine	0.8	0.8	3.9
Isoleucine	2.8	2.4	2.3
Leucine	7.6	8.1	1.6
Tyrosine	2.3	2.3	3.9
Phenylalanine	3.8	4.4	3.1
Histidine	1.5	1.2	3.9
Lysine	6.1	6.2	6.2
Arginine	5.8	6.8	7.8
Tryptophan	3.7 ^b	1.6	

^a Value obtained after oxidation of protein with performic acid prior to acid hydrolysis.

^b Determined by spectrophotometric method.

TABLE III

Effect of different cations on the activity of hmRNase

Assays were carried out with *E. coli* [³H]RNA substrate as described under "Experimental Procedures." Percentage activity retained by the enzyme in the presence of various ions, calculated with respect to the activity observed in control without the added ions is presented.

Cation	Concentration	Activity remaining
	mM	%
Mg ²⁺	2	95
	5	50
	10	0
Mn ²⁺	2	2.6
Ca ²⁺	2	2.0
Fe ³⁺	1	60.3
Fe ²⁺	1	0
Cu ²⁺	1	0
Zn ²⁺	1	0
Co ²⁺	1	59.9

85% loss of activity was observed. The enzyme was totally inactivated by heating at 90 °C for 10 min.

pH Profile—The hmRNase was assayed in HEPES buffer (pH range of 6.5–8.5) with *E. coli* [³H]RNA as the substrate. The enzyme showed a broad pH optimum in the range of 7.5–8.0 (not shown). pH optimum in the alkaline range is a characteristic of the secretory RNases (1). The isoelectric point of hmRNase was found to be 6.8 as judged from the mobility on an isoelectric focusing gel (not shown).

Effects of Different Cations—Various cations were tested for their effect on hmRNase (Table III). Sodium chloride (50 mM) was found to increase the activity to a slight extent (and hence was routinely used in reaction buffer). However, at higher concentrations of Na⁺ (above 0.15 M) there was inhibition of the enzyme activity (data not shown). Except for Mg²⁺ all other cations in the 1–2 mM range inhibited the activity tested on *E. coli* [³H]RNA.

Time Course of the Reaction—The reactions of hmRNase

showed a lag at the initial time points with *E. coli* RNA and synthetic ribopolymers as substrates (data not shown). A lag of 5 min was observed when 3.5 mg/ml enzyme was used in a reaction with 1 mg/ml substrate. The time lag decreased at higher concentration of the enzyme (7 mg/ml). To test if hmRNase was "activated" during incubation, it was preincubated at 37 °C for 15 min in the reaction buffer prior to the addition of RNA. This did not abolish the lag, and the time course at the two concentrations tested remained identical to that without preincubation (data not shown).

Effect of Mononucleotides on hmRNase Activity—Certain mononucleotides were tested for their effect on hmRNase activity, since they are either the products or product analogues during the reaction. Mononucleotides 5'-CMP, 5'-GMP, dGMP, ATP, and dATP at a concentration of 0.5 mM stimulated the reaction 2- to 3-fold. At lower concentration of the nucleotides (0.1 mM) there was no change in the level of activity (Table IV).

Action on Natural Substrates—Various naturally occurring substrates such as rRNA, tRNA, and poly(A⁺)-RNA were tested for their degradation by hmRNase. All of them were degraded equally efficiently at high concentrations of the enzyme (800 µg/ml). However, at low enzyme concentrations (100 µg/ml), mRNA was degraded to a greater extent, and the degradation level decreased in the order: mRNA (50.4%), rRNA (36.1%), and tRNA (20.5%). High molecular weight avian myeloblastosis virus RNA was completely degraded in 15 min at 37 °C (not shown).

Action on Synthetic Homopolymers—hmRNase was found to be a pyrimidine-specific enzyme. While the degradation of poly(rC) and poly(rU) were 73.5 and 74.6%, respectively, in 30 min, for poly(rA) and poly(rG) the degradation was 5.3 and 1.5%, respectively. When the double stranded substrate, poly(rU)·poly(rA) (both strands labeled separately) was used as a substrate the pyrimidine strand was selectively degraded, while the purine strand remained intact. Poly(rU) annealed with poly(dA) was more susceptible to degradation compared to poly(rA)·poly(rU) or single stranded poly(rU) (Fig. 4). The effect of adjacent purine and pyrimidine bases on the cleavage of the phosphodiester bond by hmRNase was examined with the dinucleotide substrates CpG and GpC. The products of degradation were analyzed by thin-layer chromatography as described under "Experimental Procedures." When distilled water was used as solvent, spots corresponding to guanosine were observed with CpG as substrate with hmRNase and RNase A. A spot corresponding to neither guanosine nor cytosine was observed with GpC as substrate. To test if GpC was not degraded or inactivated in any way, it was reacted with crude snake venom phosphodiesterase. This resulted predominantly in a spot corresponding to guanosine and a

TABLE IV

Effect of mononucleotides on hmRNase activity

Assay was carried out in the presence of mononucleotides in a standard assay with *E. coli* [³H]RNA as a substrate at 37 °C for the given time. Control was without any additional nucleotides.

Nucleotides	% substrate degraded			
	10 min		20 min	
	0.1 mM	0.5 mM	0.1 mM	0.5 mM
Control	19.5	19.5	48.3	48.3
5' CMP	18.8	66.8	36.8	88.9
5' GMP	20.5	64.5	48.1	83.1
5' AMP	24.0	26.9	48.3	42.6
ATP	27.7	65.1	47.2	82.1
dATP	19.3	66.8	34.1	83.7
dGMP	32.7	61.5	48.5	81.0

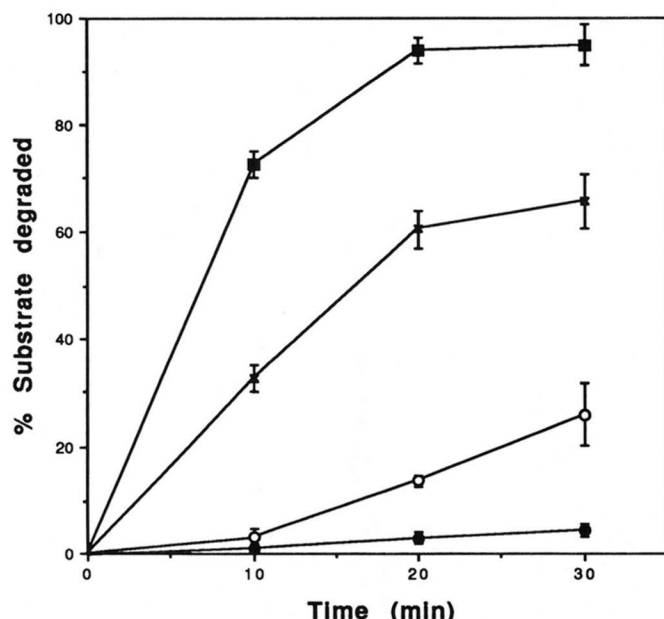


FIG. 4. Degradation of pyrimidine strand in different substrates by hmRNase. RNase assay on poly[³H]rA·poly(rU) (●), poly(rA)·poly[³H]rU (○), poly[³H]rU (×), poly(dA)·poly[³H]rU (■), was carried out as described under "Experimental Procedures."

minor spot corresponding to cytosine, perhaps due to the contaminating phosphatases in the enzyme preparation. When the same sheet was chromatographed in 0.075 M Tris. HCl (pH 8.0), the spots corresponding to mono- and dinucleotides migrated indicating that material remaining at the origin with water as solvent was not due to UV-adsorbing contaminants. These results indicate that hmRNase cleaved 3' to a pyrimidine and acted on CpG to yield Cp and G similar to RNaseA, whereas GpC was resistant to degradation (not shown).

Effect of Substrate Concentration on hmRNase Activity—Different concentrations of poly(rC), poly(rU), poly(rA)·poly(rU), and *E. coli* RNA were studied using different assay conditions as described under "Experimental Procedures." Reactions were carried out in the linear range with respect to the time of incubation and the enzyme concentration for each of the substrates. In all these reactions, at lower substrate concentrations (up to 2.5 μ g/ml for poly(rC); 25 μ g/ml for poly(rU); 5 μ g/ml for poly(rA)·poly(rU); and 450 μ g/ml for *E. coli* RNA) the rate of reaction did not increase in proportion to the substrate concentration. Furthermore, at high substrate concentrations a sharp decrease in the rate of degradation was observed (above 15 μ g/ml for poly(rC); 100 μ g/ml for poly(rU); 10 μ g/ml for poly(rA)·poly(rU); and 4.5 mg/ml for *E. coli* RNA). Between these two ranges, the rate of reaction increased with substrate concentration (data not shown). Hill plots were obtained for different substrates using data points where no inhibition was observed (excluding the points at high substrate concentration where the reaction was inhibited). The Hill coefficients were calculated for poly(rC), poly(rU), poly(rA)·poly(rU), and *E. coli* RNA and are 3.6, 2.5, 1.2, and 1.3, respectively.

Absence of Immunological Cross-reactivity with Human Serum Proteins—Ouchterlony double diffusion analysis was performed using antiserum against hmRNase in the central well and samples of human sera and milk plasma in the peripheral wells. Fig. 5 shows no cross-reactivity between anti-hmRNase antibodies and the samples of blood serum. This observation suggests that the hmRNase is immunologically distinct from any of the human serum proteins and is

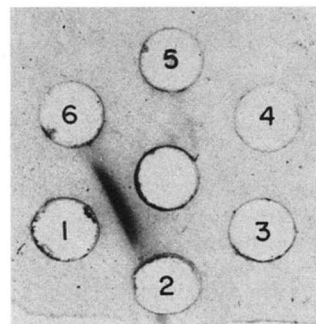


FIG. 5. Ouchterlony double diffusion analysis. The Ouchterlony double diffusion was carried out in 1% agar-agar gel plates. Rat antiserum against purified hmRNase was placed in the central well. Other samples loaded in peripheral wells are: 1, human milk plasma; 2, preimmune rat serum; 3, 2-fold diluted human serum sample No. 1; 4, undiluted human serum sample No. 1; 5, 2-fold diluted human serum sample No. 2; 6, undiluted human serum sample No. 2.

produced in the mammary tissues rather than being transported from blood.

DISCUSSION

Identification of a High Molecular Weight Ribonuclease—In human milk there are at least two proteins having RNase activity; one of them has a molecular mass of 80 kDa and the other 14 kDa (Fig. 1). Earlier, Dalaly *et al.* (27) described the purification of two ribonucleases from human milk with a molecular mass of about 14 kDa. It is likely that these RNases correspond to the 14-kDa RNase observed on the zymogram in this study. Present investigations are directed toward the analysis of the 80-kDa ribonuclease (hmRNase). Usually RNases observed in the body fluids range in their molecular mass from 13.7 kDa for human urine RNase to 45 kDa for RNase from human serum (3, 28–30). However, the human serum RNase of 45 kDa was shown to aggregate, resulting in species up to 150 kDa in molecular mass (28–30). Liu and Owens (31) observed rat milk RNases eluting in a broad peak on a Sephacryl column, part of which had a molecular mass corresponding to 80 kDa.

The hmRNase seems to be distinct from the low molecular mass RNase present in milk plasma. Immunoblotting of proteins in crude milk plasma with anti-hmRNase polyclonal antibodies did not recognize the low molecular mass enzyme, suggesting that it is not a degradation product of hmRNase (Fig. 3C).

Substrate Specificity—hmRNase shows a specificity for pyrimidines as indicated by the degradation of poly(rC) and poly(rU) by hmRNase and the resistance of poly(rG) and poly(rA) homopolymers. It is thus similar to RNase A and other secretory RNases present in body fluids (1, 32–34). However, unlike RNase A, hmRNase acts on double stranded substrates, poly(rA)·poly(rU) and poly(dA)·poly(rU). The pyrimidine specificity was maintained in a double stranded substrate like poly(rA)·poly(rU), where poly(rU) was selectively degraded (Fig. 4). It is interesting that poly(rU) present in a poly(dA)·poly(rU) complex was digested by hmRNase to a significantly higher extent compared to poly(rU) or poly(rA)·poly(rU), which suggests that hmRNase degradation of pyrimidine strands varies for different structural contexts. The cleavage pattern with the synthetic dinucleotide, CpG and GpC, shows that the diester bond 3' to a pyrimidine, but not to a purine is cleaved; a cleavage typical of a phosphotransferase mechanism as in the case of RNase A (32).

The structural features of the substrates appear to have an effect on the hmRNase activity at low enzyme concentrations.

The extent of degradation was found to be in the order tRNA < rRNA < mRNA, a preference probably related to the secondary structure(s) of these RNAs. This difference could be due to either binding of RNA to the enzyme or in the subsequent catalytic steps following binding. However, at high enzyme concentrations all these substrates were degraded to comparable extents (90%). Some mammalian RNases are known to exhibit preferences in their action toward a structure or limited nucleotide sequence such as for double strandedness (35) or for dinucleotides (36–38).

Kinetic Behavior—Kinetic studies of hmRNase using poly(rU), poly(rA)·poly(rU), poly(rC), and *E. coli* RNA indicate a non-Michaelis-Menten behavior. Hill coefficients for poly(rC) and poly(rU) were 3.6 and 2.5, while for poly(rA)·poly(rU) and *E. coli* RNA were 1.2 and 1.3, respectively. Non-hyperbolic kinetics were also reported for RNase A (39–41) and the dimeric seminal RNase (42–44). In the case of RNase A, Walker *et al.* (39) suggested that a sharp decrease in the initial rate of reaction observed at substrate concentrations of 30–50 mM cyclic CMP could be due to transition from one enzyme form with low K_m to another form with high K_m . Multiple interactions of the substrate with the enzyme were also suggested to result in the nonhyperbolic kinetics (41–44). For RNase A, the interaction of polynucleotides with the enzyme was shown to involve a series of 8–9 charged groups. The binding to polynucleotides spanned a large portion of the surface including the cleft of the catalytic center (45). In our study where polynucleotides were employed, it is difficult to dissect the changes caused by multiple interactions of the substrate with the enzyme at catalytic site *versus* other non-catalytic sites.

A nonlinear time course was observed in the hmRNase reaction (data not shown). The initial lag followed by a spurt in the reaction could be due to one or more of the following reasons: (i) initially, the substrate could be cleaved into large products that would not be detectable under our assay conditions; (ii) self-activation of the enzyme during incubation at 37 °C; (iii) stimulation of the enzyme by the product(s) of the reaction; (iv) slow decrease in substrate concentration leading to the elimination of inhibitory effect at that substrate concentration (assuming substrate inhibition). The second possibility seems to be unlikely since preincubation of the enzyme at 37 °C in the reaction buffer prior to the assay did not eliminate the lag. On the other hand, a 2–3-fold increase in the RNase activity was observed by the addition of mononucleotides (at 0.5 mM concentration, see Table IV) which are the likely products or their analogs. The first and the fourth possibility listed above cannot be ruled out since the increase in the enzyme substrate ratio reduces the lag time. Nonlinearity in the time course was also reported for crude human serum RNase with poly(rC) as substrate (46).

Origin of hmRNase—Whether the milk RNases are synthesized and secreted by the mammary gland or transported from the blood is not yet clear. Bingham and Zittle (47) demonstrated that RNases from bovine milk were identical to bovine pancreatic RNase in chromatographic properties and amino acid composition. Immunological cross-reactivity was observed between these RNases (48) which suggested the transport of bovine milk RNase from blood. However, Liu *et al.* (49) and Liu and Owens (31) have suggested the mammary origin of rat milk RNase, based on a unique stimulation of milk-RNase by Ca^{2+} in contrast to the serum RNase. In immunological studies using anti-hmRNase antibodies, no cross-reacting species could be detected in human sera (Fig. 5). Also, the size of the hmRNase makes it quite distinct from human serum RNases reported so far. These observations

would suggest the site of synthesis of hmRNase to be mammary tissues.

A study using monoclonal antibodies obtained against hmRNase has revealed some unexpected findings (24). hmRNase shares several physical, chemical, and antigenic properties with the major species of lactoferrin, an 80-kDa iron-binding glycoprotein found in high concentrations in milk (50). The eight NH_2 -terminal amino acid residues of the hmRNase are identical with the published sequence of lactoferrin (24). However, the hmRNase differs from lactoferrin in the possession of potent nuclease activity and in the lack of any significant iron binding activity. In fact, ferric and ferrous ions inhibit RNase activity (Table III). A comparison of the amino acid composition of hmRNase with that derived from the published sequences of human lactoferrin reveals many similarities. However, differences are observed for the percentages of cysteine, proline, arginine, valine, and tryptophan (Table II). hmRNase is quite distinct in amino acid composition from human pancreatic RNase. Further studies are underway to define the differences between lactoferrin and hmRNase.

Purification and characterization of milk RNase was the first step in our endeavor to analyze the role of milk RNase in mammary tumorigenicity. Monoclonal antibodies against hmRNase would be useful in immunohistochemical staining of large numbers of tissue samples from breast cancer patients. Such a study will provide information on whether RNase expression correlates with mammary tumorigenicity. Monoclonal antibodies will also be used for accurate quantitative and qualitative analysis of hmRNase from individual donors to continue our studies on the significance of low levels of RNase activity seen in the milk from Parsi women and higher incidence of mammary tumors observed in this community (14–16).

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