

RIBONUCLEASE IN BOVINE MILK

Elizabeth W. Bingham and Charles A. Zittle

Eastern Regional Research Laboratory^{1/}
Philadelphia 18, Pennsylvania

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The ribonuclease of milk has not been investigated, although there has been in recent years a rapid expansion of our knowledge of ribonuclease in body fluids such as cerebral spinal fluid (Houck, 1958), blood (Levy and Rottino, 1960; Rabinovitch and Dohi, 1957; Zittle and Reading, 1945), urine (Levy and Rottino, 1960), and the extracellular fluid of skin (Tabachnick and Freed, 1961).

Zittle and DellaMonica (1952) noted that certain purified fractions of bovine milk showed phosphodiesterase activity when ribonucleic acid was used as the substrate. Bailie and Morton (1958) showed that the nucleic acid content of mammary gland microsomes diminished when the microsomes were incubated in milk serum for 12 hours, and suggested that the phosphodiesterase of Zittle and DellaMonica (1952) might have caused the decrease in nucleic acid. They suggested that milk microsomes (with a low nucleic acid content) might be derived from mammary gland microsomes, which contained a much higher nucleic acid content.

In this report evidence is presented for the presence of relatively high concentrations of ribonuclease in cow's milk. Some properties of this enzyme, as well as its partial purification, are described.

Materials and Methods

Milk was purchased from a local dairy.

Ribonuclease was determined by the method of Tabachnick and Freed (1961). The assay mixture contained 0.65 ml Michaelis' veronal-acetate buffer (pH 7.5),

^{1/} Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

0.25 ml 1% purified yeast nucleic acid and 0.1 ml enzyme. After 5 minutes incubation at 38° C, the reaction was stopped by the addition of 3 ml glacial acetic acid-tertiary butanol (1:2). After standing at 4° C for at least 1 hour, the mixture was centrifuged at 4° C for 10 minutes at 2500 X G. The supernatant was diluted 1:6 with water. The optical density was then read at 260 mμ in a Beckman Model DU spectrophotometer*, using a 1 cm light path. Readings were corrected for enzyme blanks. The net optical density of the final diluted active reaction mixture was kept below 0.280 optical density. Up to this value the ribonuclease activity was proportional to the quantity of ribonuclease assayed with both milk samples and with crystalline pancreatic ribonuclease (Sigma lot R718-202*). The increase in optical density, which is a measure of ribonuclease activity, was linear with time for at least 15 minutes.

Results are expressed as μg of crystalline ribonuclease. The optical density reading of the final diluted mixture multiplied by a factor of 0.3 represented the μg of ribonuclease assayed. Specific activity is expressed as mg of crystalline ribonuclease equivalent per mg of total protein.

Protein was measured by means of the absorption at 280 mμ.

Phosphodiesterase activity was measured using calcium [bis(p-nitrophenyl) phosphate]₂ as the substrate, following the procedure of Sinsheimer and Koerner (1952).

Results

Table 1 shows the ribonuclease present in various milk fractions. The high value for whey indicates that very little, if any ribonuclease precipitates with the casein. Although the value for cream is very close to the value for skim milk, cream represents only 14% of the volume of whole milk.

The results summarized in Table II indicate that the ribonuclease activity of milk is high compared with values reported for other body fluids.

* It is not implied the U. S. Department of Agriculture recommends the above company or its product to the possible exclusion of others in the same business.

Table I
Ribonuclease in Milk

Sample Tested	$\mu\text{g/ml}$
Milk, Skim	24.9
Milk, pasteurized, skim	25.5
Milk, pasteurized, homogenized	25.5
Cream	27.6
Whey, rennin	26.7
Whey, acid (pH 4.6)	32.7
Whey, acid (pH 4.0)	31.5

Table II
Ribonuclease in Body Fluids

Sample	$\mu\text{g/ml}$	Reference
Human blood serum	.18 - .24	Levy and Rottino (1960)
Human urine	.53 - .77	" " " "
Guinea pig blood serum	7.5	Rabinovitch and Dohi (1957)
Rat blood serum	.75	" " " "
Rat urine	1.2	" " " "
Bovine milk	25.0	Table I

The activity of the milk ribonuclease was measured in acetate-veronal buffers (Michaelis, 1931) ranging from pH 4.2 to pH 8.5, all of which were of constant ionic strength ($\mu = .17$). The pH optimum is 7.5 and falls sharply on both sides of the optimum (Fig. 1). The pH optimum is the same as that reported for crystalline pancreatic ribonuclease (Kunitz, 1940).

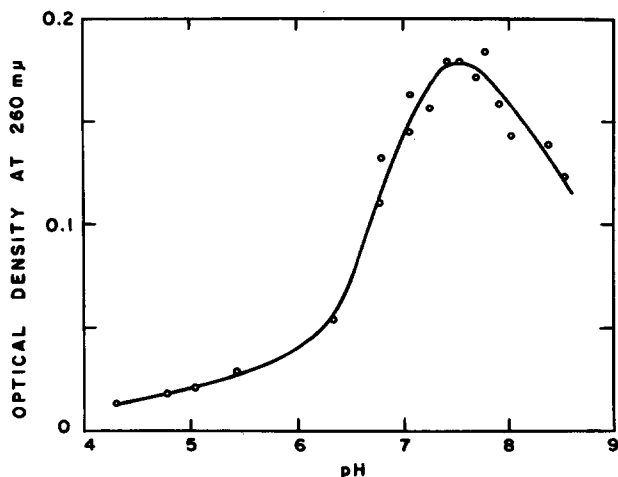


Figure 1.

Effect of pH on hydrolysis
of ribonucleic acid by skim milk.
The milk was diluted 1:50 before
assaying.

Crystalline pancreatic ribonuclease is stable to heat in the range of pH 2 - 4.5, but is inactivated by heat at higher pH values (Kunitz, 1940). The milk ribonuclease seems to resemble the pancreatic enzyme in this respect. Because the casein of milk precipitates at acid pH values, the whey was used to check the stability of milk ribonuclease. The casein was removed by adjusting the milk to pH 4.6 and filtering. Samples of whey were adjusted to pH 3.5 and 7.0, respectively. Aliquots at both pH values were heated for 20 minutes at 90° C and then rapidly cooled. The whey heated for 20 minutes at pH 3.5 retained all its ribonuclease activity. The ribonuclease of the pH 7.0 whey lost 50% of its activity after 5 minutes heating and 100% after 20 minutes heating.

Preparations with a specific activity 300 times greater than that of the skim milk have been obtained by the following procedure. One liter of raw skim milk was stirred vigorously with 1 liter of water and 20 g of IRC-50 in the NH_4^+ form, prepared by the procedure of Morrison *et al.* (1957). The resin was collected and washed on a sintered-glass funnel with copious amounts of distilled water. When the filtrate was clear, the resin was eluted with 15 ml portions of 1 M NaCl. This elution was repeated 3 times. The fourth elution contained negligible ribonuclease activity.

The eluates were combined and cooled to 4° C. Acetone (4° C) to 46% concentration was added to the eluate with stirring. The precipitate was centrifuged at 4° C and discarded. To the supernatant, cold acetone was added to a final concentration of 66%. After centrifuging at 4° C the precipitate was dissolved in distilled water. The specific activity of the purified ribonuclease indicates that 1 mg of total protein has the activity of 0.32 mg of crystalline pancreatic ribonuclease; that is, on this basis it has about one-third the activity of crystalline ribonuclease. Preliminary experiments indicate that the enzyme can be further purified by column chromatography. The summary of the purification procedure is shown in Table III.

Table III
Purification of Ribonuclease from 1000 ml Milk

Sample	Ribonuclease (Total in mg)	Protein mg	Specific Activity mg/mg
Milk	31.9	33,400	.00096
Eluate	12.7	288	.044
46-66% Acetone ppt.	12.0	37	.32

Although skim milk showed phosphodiesterase activity using $\text{Ca}[\text{bis}(\text{p-nitrophenyl})\text{phosphate}]_2$ as a substrate, no phosphodiesterase could be found in the fraction eluted from the IRC-50.

Acknowledgment

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