

Crystalline Ribonuclease from Bovine Milk

Bingham and Zittle (1) isolated ribonuclease from bovine milk using IRC-50 resin and found it to be identical to pancreatic ribonuclease A. In separating the red protein and lactoperoxidase from milk with DEAE cellulose and phosphocellulose adsorbents (2), ribonuclease was obtained as a by-product. Since milk is a good source of ribonuclease, this report will deal with another method for its isolation. Also, starting with a small amount of material, milk ribonuclease was crystallized and its disc electrophoretic properties determined.

The fractionation of milk from a single cow and the chromatographic procedures for isolating ribonuclease followed the methods described for the isolation of the iron-binding proteins (2). The two fractions eluted from the phosphocellulose column with 0.1 M phosphate-0.05 M NaCl, pH 6.5, and 0.1 M phosphate-0.1 M NaCl, pH 6.9, were greenish in color and contained lactoperoxidase and ribonuclease. The proteins were recovered from these two fractions and resolved on a Sephadex G-200 column 2 by 70 cm previously equilibrated with 0.1 M phosphate, pH 7.0, at 3 degrees. The ribonuclease was eluted in a separate peak following the lactoperoxidase. Disc electrophoresis of this fraction at acid pH (7) showed three bands, a heavy faster-moving zone corresponding to ribonuclease A and two minor slow bands. Milk fractions from a number of cows were examined and gave this typical pattern in which the mobilities of the major faster protein were identical.

Ribonuclease was crystallized using a modification of the method of Kunitz (5). The recovered protein (65 mg) from the ribonuclease fraction after separation on Sephadex was dissolved in 0.33 ml water and made to about 33% saturation by the slow addition of 0.16 ml saturated ammonium sulfate previously adjusted to pH 4.05. The solution, pH 5.6, became turbid and was centrifuged (25 degrees). After adjusting the supernatant to pH 4.1 with 0.04 ml of 0.5 N HCl, it became turbid but no crystals formed. With the addition of 0.03 ml saturated ammonium sulfate, pH 4.05, the solution became very viscous. Next day, 0.02 ml

water was added and after a time rectangular crystals were observed under the microscope. Also, the solution was no longer viscous and showed strong birefringence. On adding 0.05 ml water and cooling the mixture to 3 degrees, most of the crystals dissolved and a small amount of precipitate was removed by centrifugation (3 degrees). The supernatant slowly became turbid on the addition of 0.02 ml saturated ammonium sulfate, pH 4.05, at 3 degrees but no crystals were observed. At 25 degrees, however, crystals were obtained. For the final recrystallization, the crystals recovered by centrifugation (25 degrees) were dissolved in 0.4 ml water and slowly made to about 40% saturation with ammonium sulfate solution. In each recrystallization, the solution went through a viscous phase before the crystals formed. The crystals were in the form of thin, rectangular plates that were easily broken. A photograph of the crystals, which resemble the thin long plates found for pancreatic ribonuclease by Kunitz, is shown in Figure 1. On recovering the protein, the combined early precipitates amounted to 20 mg, the crystals 20 mg, and the crystal supernatant 12 mg. Figure 2, A shows disc electrophoretic patterns of the three fractions, together with commercial preparations of bovine pancreas ribonuclease (5 \times crystallized) and ribonuclease A. On crystallization of the milk ribonuclease fraction, the minor proteins are precipitated with the early fractions (Figure 2, A-1). Only a slight smear of slower-moving protein is found in the crys-

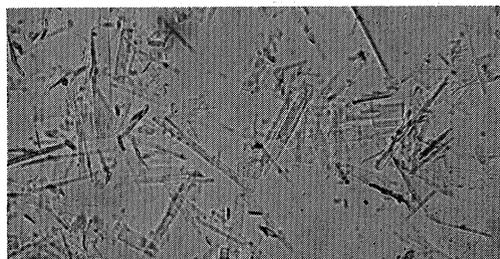


FIG. 1. Crystalline bovine milk ribonuclease.

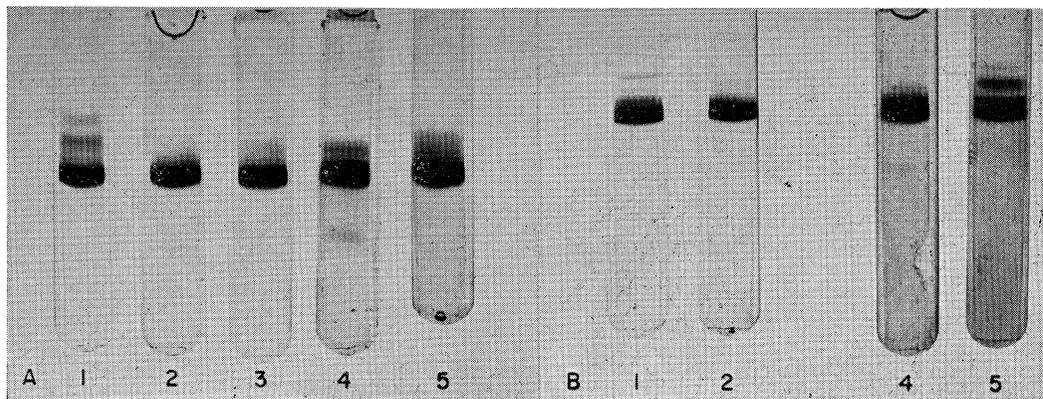


FIG. 2. Disc electrophoretic patterns of the three fractions obtained from the crystallization of milk ribonuclease and commercial ribonuclease. A: 1) precipitated fraction; 2) crystalline fraction; 3) supernatant fraction; 4) 5 \times crystallized (bovine pancreas) ribonuclease; 5) ribonuclease A (bovine pancreas). B: Same as A only in 8 M urea.

talline and supernatant fractions (Figure 2, A-2, 3), whereas more smearing is shown with commercial ribonuclease A (phosphorus-free) (Figure 2, A-5), which probably represents aggregates resulting from aging and storage, since the preparation was purchased in 1963. For comparison, a recently purchased sample of 5 \times crystallized ribonuclease is also shown (Figure 2, A-4). Disc electrophoresis in 8 M urea has the effect of sharpening some of the minor bands and changing their number and mobility in some instances (Figure 2, B).

Ribonuclease activity was determined at pH 5.0 by the procedure of Kalnitsky et al. (3). A unit of ribonuclease activity is defined as an absorbancy increase of 1.0 at 260 $m\mu$ after multiplying by the dilution factor. The protein concentration was determined from the absorbance at 280 $m\mu$ in 1-cm quartz cells. An extinction coefficient of 7.1 for a 1% solution was used (4). (I am indebted to Mrs. Bingham for the analysis.) The crystalline ribonuclease (milk) contained 2,210 units per milligram, whereas two commercial preparations from bovine pancreas: 5 \times crystallized ribonuclease and ribonuclease A, both showed identical activities of 2,613. The lower activity found for the crystalline protein (milk) compared to the commercial preparation (pancreas) cannot be related to differences in their electrophoretic patterns.

The amino acid composition of the milk crystalline ribonuclease shows good agreement with

values obtained from milk ribonuclease and ribonuclease A (1, 6).

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