# CRYSTALLINE RIBONUCLEASE FROM BULL SEMEN °

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The present communication deals with the crystallization of bull semen ribonuclease and with some tests of purity on the enzyme crystals. This enzyme, has been isolated in our laboratory (D'Alessio and Leone, 1963) and shown to be different from the homologous pancreatic enzyme, on the basis of the respective sedimentation velocities (Forlani et al. 1967) as well as of the elution patterns from SE-Sephadex C-50 and from Sephadex G-75 (Floridi and D'Alessio, 1967). Molecular weight has been correspondingly calculated to be 25,500 for seminal RNase, as against the known value of 13,680 for pancreatic RNase. Until now, the purest preparation was obtained after column chromatography on SE-Sephadex C-50, which yields an enzyme of good specific activity (27-33 Kunitz Units/mg), homogeneous in the ultracentrifuge. Recently a further purification step has been introduced, represented by a second chromatography on a column of BioRex 70, in the $Ca^{++}$ form (a manuscript is in preparation on the whole purification procedure).

## METHODS AND MATERIALS

Ribonuclease activity was determined by the spectrophotometric method of Kunitz (1946), protein by the biuret method of Gornall et al. (1949). The

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micro-immunodiffusion technique was effected on microscope slides, 2.5x7.5cm, on which 1% agar (Special Agar-Noble, Difco) in 0.15 M Na phosphate buffer pH 7.4 had been stratified. Both the antigen ( crystallised enzyme ) and the purified antibodies ( prepared from rabbit antiserum according to Levy and Sober, 1960) were dissolved in the same phosphate buffer as above. Polyacrylamide gel electrophoresis was made at pH 4.3 according to Reisfeld et al. (1962). Glass-distilled water was used throughout.

## EXPERIMENTAL

The purified enzyme, i.e. the fraction eluted from the BioRex 70 ( $Ca^{++}$ ) column, with specific activity 40-43 K.U./mg, after dialysis against cold $H_2O$ and lyophylization, is dissolved in $H_2O$ up to 15-20 mg per ml and pH is adjusted to 4.1-4.2 with M $H_3PO_4$ solution (from here onwards pH is no longer adjusted).

To this solution, solid ammonium sulfate is slowly added at room temperature, 520 mg for every ml of solution. A faint precipitate, which forms at once, is discarded by centrifugation; to the clear supernatant more solid salt is added, so as to reach a final saturation of about 80%. At this point the solution, which is kept in a stoppered centrifuge tube, becomes cloudy; after 3 to 5 days at room temperature and in the dark (there is no hint, however, of darkness being a necessary condition for crystallization) the characteristic silkiness appears, and crystals are visible at the microscope. After another 24-48 hr period the crystals have grown so as to be seen by the naked eye; they are centrifuged off at 10.000 g, redissolved in $H_2O$, dialysed against $H_2O$ and lyophylised.

The yield of the crystals is between 60 and 70% of the initial purified preparation, on a protein basis; specific activity is higher in the crystals (48-50 K.U./mg) although the difference with the initial preparation (40-43 K.U./mg) is not impressive, due to the high level of purification already reached at that stage. From 100 ml of bull seminal plasma it is possible to obtain from 30 to 45 mg of crystalline enzyme. The lyophylised preparation is stable in the cold for some months at least.

The initial concentration of enzyme (15-20 mg/ml) prior to crystallization, although not critical, appears to be optimal; beyond these values gel

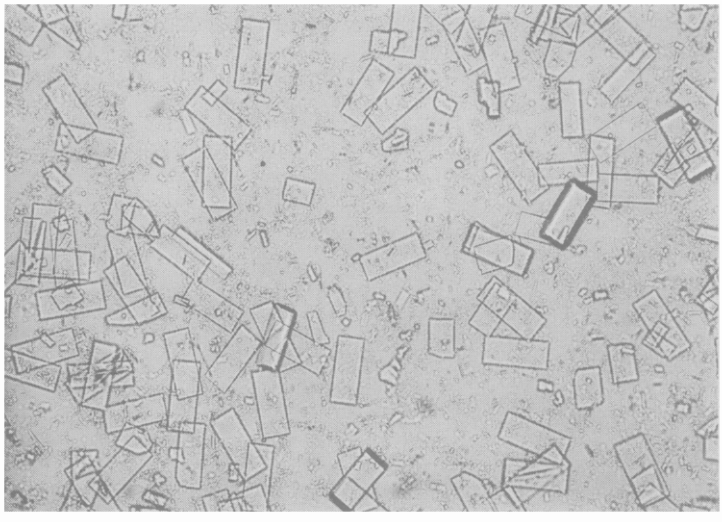

Fig. 1 - Crystals of bull semen ribonuclease (magnification x 190).

formation is often observed during addition of ammonium sulfate. In these cases, repeated additions of some 5-10 µl $H_2O$ are enough to make the gel dissolve. In any avent a useful trick is to add, when the silkiness has first appeared, still more ammonium sulfate, up to 82% saturation; in this way crystallization is speeded up, is more massive, and the yield is in excess of the 70% mentioned before.

The crystals (Fig. 1) appear like well defined, birefrangent platelets, and are quite stable in ammonium sulfate solution. Repeated crystallizations are easy to perform, after solution of the centrifuged crystals in $H_2O$ (15-20 mg/ml) to which $H_3PO_4$ has been added until pH 4.1-4.2 is reached; ammonium sulfate is then added until a definite turbidity developd; usually after 1-2 days crystallization sets in.

A few tests have been performed to check the purity of the crystallised enzyme. The ultracentrifuge pattern is symmetrical and homogeneous (Fig. 2), and immunochemical analysis as well shows a high degree of purity of the en-

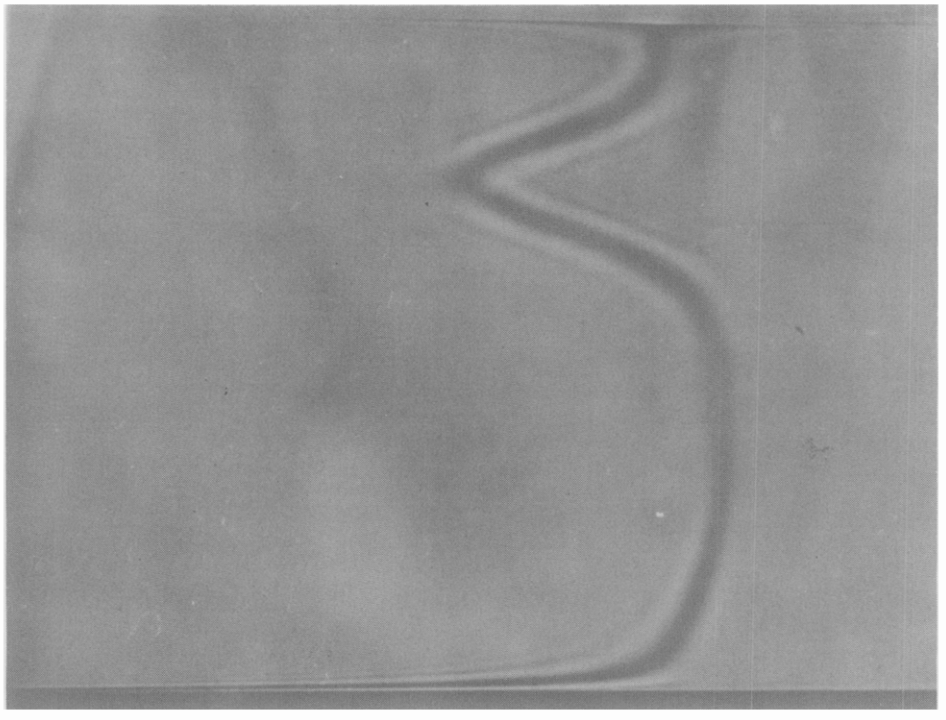

Fig. 2 - Sedimentation velocity pattern of seminal ribonuclease. Photograph taken at an angle of 40°, 160 min after reaching the full speed of 59,780 r.p.m., using a Spinco model E ultracentrifuge equipped with rotor temperature indicator and control unit. Enzyme, mg 4.98/ml, in 0.05 M sodium phosphate buffer pH 7.0; 6° C.; $S_{20,w} = 2.70$.

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zyme and absence of cross-reaction between RNase A and the rabbit antiseminal RNase serum (Fig. 3). On the other hand, when large amounts (100-200 $\mu$g) of crystalline seminal ribonuclease are analysed by polyacrylamide gel electrophoresis a minor, slower moving component is visible, which is not seen when smaller amounts (30-50 $\mu$g) are applied. Purification from this impurity has been achieved by separation on a column of phosphocellulose ( Cellex-P, Bio-Rad), elution from which is effected by a ionic strength linear gradient (Fig. 4). The minor component is eluted first and shows no activity, en-

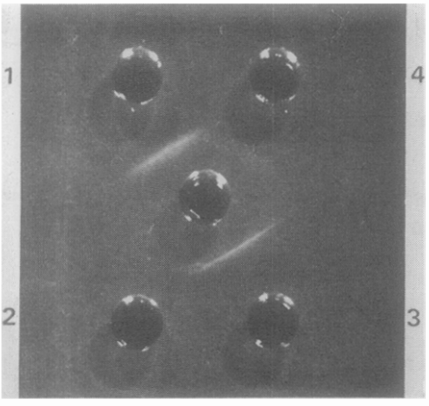


Fig. 3 - Microimmunodiffusion test. In wells 1 and 3 were deposited 10 µl each of a 0.1% solution of crystalline seminal ribonuclease in 0.15 M sodium phosphate buffer, pH 7.4; in wells 2 and 4 were deposited 10 and 20 µl respectively of a 0.1% solution of pancreatic RNase A in the same buffer. In the centre well, 15 µl (400 µg) of a solution in the same buffer of $\gamma$-globulins from rabbit antiseminal ribonuclease serum. All wells were completely filled, if required, with the sodium phosphate buffer. The $\gamma$-globulins were deposited in the centre well about 30 min before the antigens. Diffusion was usually complete in 24 hr at room temperature.

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zymatically or immunochemically, representing about 5% of the total protein. The major fraction, which forms a symmetrical, homogeneous peak, is eluted at 0.64 molarity of the ammonium formate buffer, pH 6.4. On electrophoresis of 100 µg of this fraction the minor component is no longer visible. (Fig. 5)

Therefore, in conclusion, the crystalline bull semen RNase purified by chromatography on phosphorylated cellulose column, appears to be pure by all the criteria utilized in this study, including the electrophoretic analysis on polyacrylamide gel.

Thus, while the number of ribonucleases isolated from a variety of tissues and animal species, as well as of microrganisms, has steadily increased du-

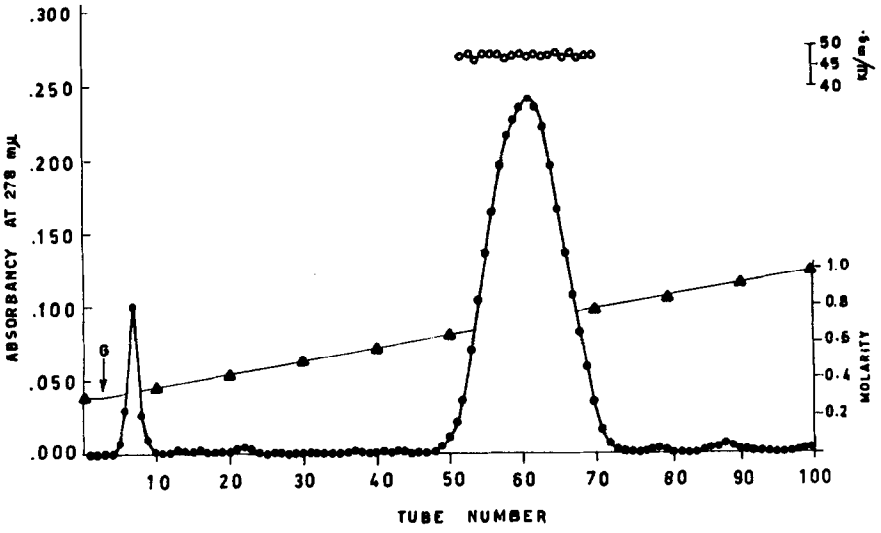


Fig. 4 – Purification of the crystallised ribonuclease on phosphocellulose. A column, 0.9 x 19 cm, of Cellex-P (BioRad) 0.85 ± 0.1 mEq/g, $NH_4^+$ form, equilibrated with 0.3 M ammonium formate buffer pH 6.4, was loaded with 15 mg of crystalline enzyme, dissolved in 2 ml of the above buffer. Ionic strength linear gradient was applied, were indicated by the arrow, by the use of a reservoir and a mixing chamber, containing each at the start 100 ml of ammonium formate buffer pH 6.4, 1.0 M the former and 0.3 M the second. Velocity, 20 ml/hr. 2ml fractions were collected; all operations at 4° C.

•——•——• $A_{278}$ mμ; o——o——o specific activity; —— Δ —— Δ —— buffer molarity.

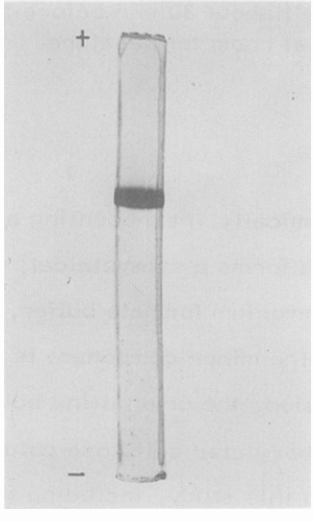


Fig. 5 – Polyacrylamide electrophoresis of seminal RNase purified on Cellex-P (100 μg). Electrophoresis was carried out for 75 min. at 4°C at 5 mA. per tube. Bottom of the picture represents cathode.

ring the last few years, the purification of the bull semen enzyme, here reported, adds one more crystalline ribonuclease to the few ones described in the literature (beef pancreas, Kunitz, 1940; Aspergillus oryzae $T_1$, Egami et al. 1964; Bacillus subtilis, Nishimura, 1960). A detailed study of the properties and of the structure of this enzyme should help to elucidate its mechanism of action.

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