Phosphatase-free Crystalline Micrococcal Nuclease*

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SUMMARY
This report relates to the preceding paper by Taniuchi and Anfinsen. Micrococcal nuclease from the strain “Foggi” has been purified from a commercially available source (Worthington). The enzyme was crystallized from an ammonium sulfate solution. The over-all purification was approximately 500-fold with an over-all yield of about 16%. The enzyme is virtually devoid of acid and alkaline phosphatases.

The preceding paper, by Taniuchi and Anfinsen (1), described a method for the preparation of crystalline micrococcal nuclease. Since we too have crystallized this enzyme, we are reporting our method, developed independently. The two laboratories have had different goals in mind. In Dr. Anfinsen’s laboratory the primary goal was to study micrococcal nuclease as a protein. While it was important to remove contaminating proteins as carefully as possible, the nature of the remaining contaminants was of no consequence. In our laboratory the enzyme was prepared to be used as a reagent in the study of nucleic acid structure. The absence of monophosphatases was therefore a sine qua non condition for our purpose.

Our previous work (2-4) on this enzyme had been done with Strain SA-B isolated from clinical material (5) and characterized in the original paper of Cunningham, Catlin, and Privat de Garille (6). The present method utilizes a different strain, “Foggi.” The crude starting material is available commercially in large quantities (Worthington). The enzyme was crystallized from an ammonium sulfate solution. The over-all purification was approximately 500-fold with an over-all yield of about 16%. The enzyme is virtually devoid of acid and alkaline phosphatases.

EXPERIMENTAL PROCEDURE

Source of Enzyme—A crude preparation of micrococcal nuclease was obtained from Worthington. It consisted of an ammonium sulfate precipitate of a medium in which Micrococcus pyogenes strain “Foggi,” was grown. Carboxymethyl cellulose, type 20, was purchased from Sigma. Crystallized serum bovine albumin was purchased from Pentex. Thymus DNA was prepared by Dr. J. Georgatsos by the method of Kay, Simmons, and Dounce (7).

Methods—Activity determinations of nuclease were performed spectrophotometrically essentially by the method of Kunitz (8), at 37° with a Gilford recorder. The substrate concentration in the final mixture (DNA, enzyme, 0.1 m Tris-HCl (pH 9.0), and 0.01 m CaCl₂) was 0.45 A₂₆₀ unit per ml. A 0.1% solution of bovine serum albumin was used routinely for dilution of enzyme.

One unit of activity was defined as a change in absorbance at 260 nm of 1.0 per min, and potency was defined as the ratio of activity units to A₂₆₀ units of the enzyme solution.
P-Nitrophenyl phosphate was used to determine both acid and alkaline phosphatases by the procedures previously described (3).

RESULTS

Initial Processing of Commercial Material—A 100-g portion of crude micrococcal nuclease (strain “Foggi,” Worthington) was suspended in 1 liter of 0.1 m Tris-HCl, pH 7.5, was stirred for 30 min at 4° and was centrifuged. The insoluble material was discarded.

The supernatant solution (“Starting material,” Table I) was diluted with 0.1 m Tris-HCl buffer, pH 7.5, and with 0.1 m EDTA (adjusted to pH 7.5) to attain concentrations of 25 x 10⁻³ m in respect to EDTA, and 10 A₂₆₀ units per ml in respect to protein.

One-liter portions in 2-liter Erlenmeyer flasks were then incubated for 15 min in a water bath at 50° and rapidly cooled on ice. A slight turbidity developed, but no attempt was made to remove it. During the period of heating a relatively selective denaturation of phosphatases occurred in the presence of EDTA. There was a loss of about 10% nuclease activity; at the same time, however, about 98% of acid phosphatase, and about 95% of alkaline phosphatase were eliminated.

Step 1—After heating and cooling to 4°, the solid ammonium sulfate was added to the liquid to attain 60% saturation. The mixture was stirred for 60 min at 0° and the precipitate was removed by centrifugation. The ammonium sulfate concentration was then raised to 75% saturation, and the mixture was again stirred for 60 min at 0°. The precipitate that formed was collected by centrifugation and dissolved in water to attain 90 A₂₆₀ units per ml.†

Step 2—Precipitation by trichloroacetic acid was performed essentially as previously described (3). The fraction obtained between 0.75 and 2.5% of trichloroacetic acid concentration,

† When kept at −20° this material may be stored for several weeks without a noticeable fall in nuclease activity.
Starting material 85,000
1. Ammonium sulfate 60-75% 13,450
2. Trichloracetic acid, 0.75-2.5% 2,284
3. Ethanol, 0-50% 531
4. Bio-Gel P-100 (Fig. 1, tubes 90-115) 159
5. Carboxymethyl cellulose (Fig. 2, tubes 203-220) 38
6. Crystals 27

*The apparent increase in activity is probably due to inhibition of the enzyme by traces of trichloracetic acid.

which contained most of the enzyme, was collected by centrifugation. It is imperative that this precipitate be dissolved in dilute ammonia and immediately adjusted to pH 7.0 with 3 M ammonia.

Step 3—Precipitation with 50% ethanol was also performed essentially as described previously (3). The active precipitate was dissolved in 0.1 M Tris-HCl buffer, pH 9.0, and the small amount of undissolved material was centrifuged off and discarded. It was found preferable to carry out Steps 2 and 3 on the same day. The nuclease preparation after Step 3 may be stored for weeks at -20°C without a noticeable loss of activity.

Step 4—The material from Step 3, containing not more than 30 A280 units per ml, was applied to a column of Bio-Gel P-100. For a column measuring 4.5 x 50 cm, the load should not exceed 500 A280 units. The elution pattern of a typical experiment is shown in Fig. 1. The contaminating phosphatases which accompanied the nuclease during Steps 2 and 3 were rather effectively removed (Fig. 1). The fractions containing nuclease activity were pooled.

Step 5—The pooled fraction from Step 4 was applied to a column of carboxymethyl cellulose, previously equilibrated with 0.1 M sodium acetate buffer, pH 6.5. The sample applied was the eluate from Step 4 (volume 130 ml; total A280 131). Elution was carried out with the equilibrating buffer. The flow rate was 80 ml per hour, 4 ml per tube; temperature, 4°C. After 114 fractions had been collected, a linear gradient with 1 liter of sodium acetate, pH 6.5, between 0.1 and 1.0 M was started. The solid line represents protein concentration expressed as A280 units per ml; ○, nuclease potency; — — , gradient.

After an actual experiment is illustrated in Fig. 2.

Step 6—To concentrate the material, the pooled fraction from the previous step was brought up to 90% saturation with ammonium sulfate. After centrifugation of the mixture, the precipitate was dissolved in a minimum of water and was passed through a column of Bio-Gel P 2, mesh 100 to 200, 2.7 x 60 cm. The fractions containing enzyme were pooled and lyophilized.
There was no loss of nuclease activity during lyophilization. The powder was dissolved in water, brought up with solid ammonium sulfate to 70% saturation (at 0°C), and carefully adjusted with ammonia to pH 7.5. The light precipitate was centrifuged down and discarded. A small amount of solid ammonium sulfate was then added, which resulted in a slight turbidity. Crystallization of the first crop was carried out for 24 hours. Further crops of crystals were obtained by consecutive increases in ammonium sulfate concentration and readjustment of pH to 7.5. The various crops of crystals (Fig. 3) showed virtually the same potency of micrococcal nuclease. Amorphous material contaminating early crystalline preparations was found to convert to crystals on standing a week or longer at 0°C. The crystalline preparation subjected to disc electrophoresis showed only traces of impurities.

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REFERENCES

Phosphatase-free Crystalline Micrococcal Nuclease
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