An Improved Method for the Purification of Staphylococcal Nuclease

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SUMMARY

Commercial staphylococcal nuclease (Foggi strain) containing small amounts of contaminating protein can be purified by chromatography on phosphorylated cellulose columns. The purified enzyme is free of acid and alkaline phosphatase activities. It is homogeneous by polyacrylamide gel electrophoresis, and antigenically pure by immunodiffusion. The \( E_{1}^{1} \) of the pure enzyme at neutral pH is 9.3 at 280 m\( \mu \) and 9.7 at 277 m\( \mu \).

Previous methods for the isolation of an extracellular nuclease of \textit{Staphylococcus aureus} have yielded material which was essentially homogeneous and suitable for physical and structural studies (1, 2). However, such preparations are contaminated with small amounts of phosphatases, which might interfere with studies of enzymatic activity and specificity. Furthermore, we have observed that these preparations are not antigenically homogeneous, but exhibit two precipitin lines when tested by double diffusion on agar plates. The present paper describes purification of the nuclease by means of a phosphorylated cellulose ion exchange resin, which yields a homogeneous preparation of the enzyme, free of phosphatase and other demonstrable contaminants. Another method of preparing a phosphatase-free crystalline nuclease was recently described by Sulkowski and Laskowski (3).

EXPERIMENTAL PROCEDURE

Staphylococcal nuclease (Foggi strain) was purchased from Worthington. It was prepared according to the procedures described by Anfinsen, Rumley, and Tanuiuchi (1) and Heins, Tanuiuchi, and Anfinsen (2). Highly polymerized salmon sperm DNA was purchased from Callbiochem. Yeast RNA and \( \beta \)-nitrophenyl phosphate were purchased from Sigma. Phosphorylated cellulose (7.4 meq per g) was a Whatman product. All other reagents used were of analytical grade.

Nuclease assays (both DNase and RNase activities) were performed spectrophotometrically as described earlier (4). Activities of acid and alkaline phosphatase in nuclease preparations were tested with \( \beta \)-nitrophenyl phosphate as substrate, as described by Ohsaka, Mukai, and Laskowski (5). The reaction mixtures were incubated at 37\( ^\circ \) for 30 min to 20 hours, and the reaction was stopped by addition of equal volumes of 0.2 M NaOH, at which time the absorbance was measured at 400 m\( \mu \). One unit of activity is equal to the release of 1 \( \mu \) mole of \( \beta \)-nitrophenol per min at 25\( ^\circ \). Specific activity is defined as units of activity per 1.0 absorbance unit at 280 m\( \mu \). The factors for converting the data from 37\( ^\circ \) to 25\( ^\circ \) are 0.58 and 0.74 for acid and alkaline phosphatase, respectively (5).

Rabbit antinuclease sera were obtained by immunization with nuclease emulsified in complete Freund's adjuvant (Difco). Two injections were given 7 days apart, and a third (booster) injection was given 30 days after the first immunization. At each injection, 3 mg of the commercial nuclease were given. The rabbits were bled at 5-day intervals, beginning 1 week after the last immunization. Antisera were examined by the precipitin test, by their ability to inactivate the enzymatic activity of nuclease, and by double diffusion in agar plates according to the method of Ouchterlony (6). The last technique was found most useful in following the purification of nuclease.

RESULTS AND DISCUSSION

Antisera prepared by immunization with commercial staphylococcal nuclease yielded two precipitin lines when tested by double diffusion against the same preparation of nuclease (Fig. 1). The outer line could not be detected when low concentrations of the antigen (less than 0.2 mg per ml) were applied to the plate. However, at higher concentrations of antigen, this precipitin line was very strong and resolved into more lines at concentrations higher than 1 mg per ml. These results suggested that the nuclease preparation might still contain small amounts of protein contaminants. This was supported by other criteria. Electrophoresis in polyacrylamide gels (7) gave two small bands in addition to the nuclease band (Fig. 2). End group analysis in some preparations showed traces of dinitrophenylamino acids.
other than alanine, which is the NH₂-terminal residue of the enzyme (7).¹ Both acid and alkaline phosphatase activities were found in low, although significant, amounts in the commercial nuclease. The particular preparation of nuclease described in this study had 2.2 × 10⁻² and 3 × 10⁻³ units of acid and alkaline phosphatase activity, respectively, per mg of protein.

Chromatography of the enzyme on phosphorylated cellulose columns, developed with a pH and ionic strength gradient, was found to be an effective way to obtain a pure preparation of nuclease, free of phosphatase activity and other contaminating proteins. Fig. 3 illustrates a typical chromatography pattern on phosphorylated cellulose. The first peak having absorbance at 280 nm (tubes 2 to 4), emerging at the exclusion volume, seems to be composed of small acidic peptides as was shown by peptide maps. This fraction has neither enzymatic nor antigenic activity and produces no observable band on electrophoresis in polyacrylamide gels. The peak, preceding the nuclease fraction (tubes 22 to 29), has no nuclease activity but gives a strong precipitin reaction with antiserum to nuclease on agar plates (Fig. 1, Well 5). This precipitin line is identical with the outer line observed with the unpurified nuclease (Fig. 1, Well 1). Electrophoresis of this fraction in polyacrylamide gels shows that it constitutes the additional bands in the nonpurified protein preparation (Fig. 2).

The nuclease, which was eluted from the column at a molarity of 0.72 and pH 6.2 (ammonium acetate buffer), appears pure by all the criteria utilized in this study. Alanine is the only NH₂-terminal amino acid found by dinitrophenylation of the prep-

¹ H. Taniuchi and C. Cusumano, unpublished data.
solutions having absorbances higher than 0.2. The activities of phosphatases were determined on the different fractions eluted from the phosphorylated cellulose column (Fig. 3), as described by Ohsaka et al. (5) and in "Experimental Procedure." Fractions which had low absorbance at 280 mp were those with a molarity of buffer (measured by conductivity meter); △—△, molarity of buffer (measured by conductivity meter); ▲—▲, pH.

Table I
Activity of acid and alkaline phosphatases in fractions eluted from phosphorylated cellulose column

The activities of phosphatases were determined on the different fractions eluted from the phosphorylated cellulose column (Fig. 3), as described by Ohsaka et al. (5) and in "Experimental Procedure." Fractions which had low absorbance at 280 mp were concentrated by lyophilization and dissolved in water to make solutions having absorbances higher than 0.2. The activities of the different samples were calculated from incubation periods giving linear changes in activity.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Acid phosphatase at pH 5.6</th>
<th>Acid phosphatase at pH 9.0</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>× 10^4</td>
<td>× 10^4</td>
</tr>
<tr>
<td>Commercial nuclease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubes 2-6</td>
<td></td>
<td>22</td>
<td>30</td>
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<tr>
<td>Tubes 7-20</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tubes 21-25</td>
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</tr>
<tr>
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</table>

glass-distilled water, and then lyophilized and dried over phosphorus pentoxide in vacuum. Aliquots of the dried, pure nuclease were hydrolyzed in a vacuum at 110° in constant boiling HCl for 20 and 70 hours. It was estimated from the amino acid analyses, and from knowledge of its amino acid composition (8), that 1 mg of the enzyme contained 6.0 × 10^2 ± 0.6 × 10^2 μmole of nuclease. The calculations were based on averages of amino acid analyses obtained from the 20- and 70-hour hydrolyses for histidine, serine, isoleucine, and phenylalanine. The calculated value, based on the chemical molecular weight of the enzyme (8) (16,800), is 5.9 × 10^2 μmole per mg.

A solution of 0.1% nuclease in 0.05 Tris buffer, pH 7.5, has an absorbance of 0.030 at 260 μm, and 0.970 at 277 μm (λ_max for nuclease at this pH (9)). These values are similar to those calculated from spectral studies of nuclease, based on its tryosine and tryptophan content (9), and lower than the one reported previously (1, 2).

The experiments described here showed separation of nuclease from residual impurities left after the purification procedure used earlier. The commercial nuclease used contained only low amounts of protein contaminations (5 to 10%). However, phosphorylated cellulose seems to be a suitable ion exchange resin for batch preparation of the enzyme. In a typical experiment, an ammonium sulfate precipitate (40 to 90% saturation) was dissolved in 300 ml of 0.15 m ammonium acetate, pH 6. The dark brown solution, which contained 200,000 units of DNase activity, was mixed with a slurry of 25 g of washed phosphorylated cellulose in the same buffer. Under these conditions nuclease adsorbs strongly to the resin, and most of the other impurities could be removed by filtering and repeated washing of the resin with 0.3 m ammonium acetate, pH 6. A final wash was carried out with 0.4 m ammonium acetate, pH 6. About 400 mg of protein, containing 220,000 units of DNase activity, were extracted from the phosphorylated cellulose cake with two washes of 1 m ammonium acetate. After elution, lyophilization, and a brief dialysis against water to remove most of the remaining salt, final purification was achieved on a phosphorylated cellulose column as described above.

References

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