Simple Method for the Preparation of Streptococcal Nuclease

STEPHEN C. MARKER AND ERNEST D. GRAY

Departments of Pediatrics and Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455

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A simple method for preparation and fractionation of the streptococcal nuclease by polyacrylamide gel electrophoresis is presented. The procedure is carried out with ammonium sulfate-precipitated supernatant fluids from cultures of beta-hemolytic streptococci grown to stationary phase. Electrophoresis on polyacrylamide gel and subsequent elution of the fractionated enzymes allows the preparation of sufficient homogeneous nuclease B for a large number of anti-nuclease B titrations.

The streptococcal nuclease represent a group of extracellular products commonly produced by many strains of beta-hemolytic streptococci. Indeed, among group A streptococci, strains which do not exhibit nuclease activity have not been described. The nuclease are at least four in number and have been designated A, B, C, and D. They are electrophoretically and immunologically distinct and are endonucleolytic in their action, producing oligonucleotides terminating in 5' phosphates. Streptococcal nuclease B is produced to the greatest extent and by the largest number of streptococcal strains (8).

In human infections with group A beta-hemolytic streptococci, antibodies are produced against the streptococcal nuclease. Titration of the level of anti-nuclease B (also referred to as anti-deoxyribonuclease B) has proven to be a clinically useful index of a recent streptococcal infection (7). As with streptolysin O, antibody responses to nuclease B occur regularly after streptococcal pharyngitis, and elevated titers are often found in patients with acute rheumatic fever and acute glomerulonephritis (1). After streptococcal impetigo or in patients with nephritis as a complication of impetigo, anti-nuclease B titers are generally high and therefore more useful than anti-streptolysin O responses which in this situation are usually feeble (3, 4).

The more general use of the streptococcal anti-nuclease B determination has been limited in the past by the difficulties of preparation of the B antigen homogenous with respect to nuclease activity (i.e., uncontaminated with the other streptococcal nuclease). The present report describes a simplified method of antigen purification that should allow a wider use of the anti-nuclease B determination.

MATERIALS AND METHODS

Preparation of extracellular nucleases. Previously frozen 1-ml cultures of S. pyogenes strain C203S (originally furnished by Allan Bernheimer) were grown for 6 to 8 hr in 5 ml of sheep blood Todd-Hewitt broth (Difco) at 37°C, and then transferred to 500 ml of Todd-Hewitt broth and incubated overnight at 37°C. The stationary phase culture was cooled in an ice bath (all subsequent operations were carried out at 4°C), and the cells were removed by centrifugation for 15 min at 10,000 × g. The supernatant fluid was made 85% saturated with (NH₄)₂SO₄ by the addition of solid (NH₄)₂SO₄ and was stirred for 1 hr.

The precipitate which formed was collected by centrifugation for 10 min at 7,000 × g. The portion of the insoluble material which floats on the surface of the ammonium sulfate solution was combined with that which sediments to the bottom of the centrifuge tube, and the clear fluid was discarded. The precipitate was dissolved in 25 to 40 ml of 0.001 M glycine, pH 9, and dialyzed for 3 hr against 15 to 20 volumes of the same buffer with several changes. After dialysis, the crude nuclease preparation was either immediately fractionated by polyacrylamide gel electrophoresis or frozen and stored at −20°C.

Fractionation of nucleases. The four streptococcal nucleases were separated by electrophoresis in polyacrylamide gel by the method of Davis (2). Gel buffer contained 1.0 ml of concentrated HCl, 8.0 g of tris(hydroxymethyl)aminomethane (Tris) base, 0.58 ml of tetramethylethylenediamine in 100 ml. A solution containing 30.0 g of acrylamide and 0.8 g methylenebisacrylamide in 100 ml was used to prepare the gels and was stored at 4°C. A 7.5% acrylamide gel was prepared by mixing 2.3 ml of gel buffer, 2.5
ml of acrylamide solution, 3.2 ml of water, and 2.0 ml of 0.4% ammonium persulfate. Gels 10 cm long were formed in lucite tubes 0.6 cm in diameter and overlaid with water to provide a flat upper surface. Samples 0.4 ml in volume containing 10% sucrose were applied to eight to ten gels, and electrophoresis was continued at 5 ma per gel until a bromophenol blue marker migrated 9.5 cm. The electrode buffer contained 0.6 g of Tris base, 2.88 g of glycine, and 0.01 M mercaptoethanol dissolved in 1 liter of distilled water. The gels were removed from the plastic tubes and uniformly sliced into approximately 2-mm segments with the aid of a template. To elute the nucleases, each gel slice was transferred to a glass tube and incubated at 4 C overnight with 0.5 ml of 0.001 M Tris buffer, pH 7, containing 0.001 M CaCl₂, and 0.001 M MgCl₂. The location of the nucleases in the gel was determined by assaying the eluates for nuclease activity, and the appropriate gel eluates were pooled.

**Assay of nuclease activity.** The nucleases were assayed by their degradation of deoxyribonucleic acid (DNA) to acid-soluble fragments by using the following method. Samples (0.05 ml) of gel eluates were transferred to glass tubes (13 by 100 mm). To these was added 0.2 ml of a mixture containing 0.001 M CaCl₂, 0.0001 M MgCl₂, 0.02 M Tris-hydrochloride (pH 7.0), and DNA, 500 µg/ml (Sigma Chemical Co., St. Louis, Mo.). The tubes were incubated at 37 C for 10 min and then transferred to an ice bath, and 2.0 ml of 0.2 M perchloric acid was immediately added. After allowing at least 10 min for precipitation of undegraded DNA, the acid-insoluble material was removed by filtration through a glass fiber filter (Reeve Angel 935 AH) using a Millipore filtration apparatus. The absorbancy at 260 nm of the filtrates was measured with a Gilford 240 spectrophotometer.

An alternative method of locating nuclease activity in the polyacrylamide gels after electrophoresis involves the action of these enzymes in decolorizing a DNA-methyl green complex entrapped in agar. Glass plates were coated with a mixture containing 0.5 mg of DNA per ml, 0.13 mg of methyl green per ml, 1.5% agar, 0.005 M CaCl₂ and MgCl₂, 0.025 M Tris-hydrochloride, pH 7.6. A concentrated stock solution of 1.3 mg of methyl green per ml of distilled water was prepared and extracted several times with chloroform until no further visible color was extractable. The aqueous solution of methyl green remaining was used in the assay. After the other components had been heated to dissolve the agar, the DNA was added. The mixture was pipetted onto glass plates and allowed to solidify. Samples of the gel eluates (25 µlitters) were spotted on the plates which were then incubated at 37 C for approximately 2 hr. If the plates were incubated for a prolonged period (overnight), trace levels of nuclease were detected which prevented easy location of major peaks of activity. The presence of enzyme is indicated by decolorization, and the appropriate gel eluates can be pooled.

**RESULTS**

The release of extracellular nuclease by cultures of strain C203S streptococcus appears to be dependent on the growth phase of the organism. Figure 1 illustrates this variation of total extracellular nuclease activity during growth of a culture inoculated with late logarithmic-phase cells. The release of nuclease activity occurs near the time of cessation of growth and rapidly increases during stationary phase. Further study has indicated that all four of the nucleases follow the same time course (Gray, *unpublished data*). The importance of allowing the organisms to grow to stationary phase for optimal nuclease preparation is evident from these results.

A typical separation of the streptococcal nucleases from an ammonium sulfate precipitate of culture supernatant fluid is shown in Fig. 2. The four peaks of nuclease activity correspond to the B, D, A, and C enzymes. All of these are well separated from one another and have been shown to be homogenous by the use of specific antisera to the four nucleases. The level of activity in the nuclease B peak when pooled from eight gels represents sufficient enzyme for over 1,000 microtitrations of antinuclease B (6).

Recovery of nuclease A was enhanced by the presence of 0.01 M mercaptoethanol in the gel eluant, suggesting the role of sulfhydryl groups in the activity or stability of this enzyme. The other enzymes were unaffected by mercaptoethanol; therefore this is routinely included in the eluant as well as the electrode buffer.

The electrophoretic mobilities of the streptococcal nucleases in 7.5% polyacrylamide gels, relative to that of bromophenol blue, are 0.61,
DISCUSSION

The streptococcal nucleases have been previously purified by column chromatography on diethylaminoethyl cellulose or by starch gel zone electrophoresis (8). Both of these techniques are effective but not as easily carried out as the polyacrylamide gel electrophoresis described in the present report. The latter procedure provides homogenous preparations of all four of the streptococcal nucleases. They are obtained in a one-step procedure from an ammonium sulfate precipitate of a crude supernatant fluid from a culture grown in commercially available media. The separation procedure requires relatively simple equipment and can be carried to completion in 2 days.

Previous preparations of these nucleases have employed supernatant fluids of cultures grown on dialysate media. This does have the advantage of minimizing extraneous proteins, but preparation of the media is a lengthy procedure. For obtaining nuclease B to be used as antigen in a specific neutralizing antibody system, growth of the organisms on Todd-Hewitt medium is perfectly satisfactory. For such usage, complete separation from the other nucleases, rather than from the other streptococcal products or from media constituents, is the important factor. The medium polypeptides are distributed throughout the gel as can be demonstrated by staining procedures and do not interfere with the electrophoresis or activity of the isolated enzyme. In addition, somewhat higher enzyme levels are attained in culture supernatant fluids of Todd-Hewitt than in dialysate medium.

The observation that maximal enzyme production occurs at the onset of stationary phase has not been observed with some other strains of streptococci (5). It has been found that this phenomenon is dependent not only on the strain of bacteria but also on the medium used to grow the organisms, being most pronounced with Pfanstiehl dialysate medium (E. D. Gray, unpublished data). To assure maximal enzyme recovery it is advisable, in any case, to allow the culture to grow overnight to stationary phase.

This method of preparing and fractionating the extracellular streptococcal nucleases has proven to be rapid, effective, and relatively simple to carry out. In a single electrophoresis run it is possible to produce sufficient nuclease B for over 1,000 antisera titrations by the micromethod of Nelson et al. (6). It should allow the widespread use of this valuable diagnostic aid.

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Fig. 2. Polyacrylamide gel electrophoresis of extracellular nucleases (DNases) of S. pyogenes strain C203S. After concentration by ammonium sulfate precipitation, the extracellular nucleases were subjected to electrophoresis on 7.5% polyacrylamide gel. Nuclease activity was measured in the eluates of the gel slices. A unit of nuclease activity represents an acid-soluble absorbance at 260 nm of 1.0 after 10 min of incubation at 37°C under the standard assay conditions as described in the text.

0.091, 0.96, and 0.41 for nucleases A, B, C, and D, respectively. This gel concentration most efficiently separates all four enzymes; however, if only nuclease B is of interest, it may be most readily separated from the other nucleases by using 4% polyacrylamide gels. In this case, the relative mobilities of nucleases A, B, C, and D are respectively 0.90, 0.15, 1.0, and 0.67. The use of 4% polyacrylamide gels thus allows the other enzymes to migrate relatively farther toward the anodic end of the gel leaving nuclease B near the top of the gel.

On electrophoresis in 4% gels or even in 7.5% gels (Fig. 2), it has been observed that nuclease B exhibits heterogeneity, with asymmetry in the activity profile. It is not yet known whether these are due to previously unresolved streptococcal nucleases or some artifact generated during enzyme isolation. It has been established that all the nuclease activity of nuclease B can be inhibited by specific antisera, but a determination of the immunologic reactivity of the individual components must await their separation.

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