Characterization of the A Component of *Streptococcus zymogenes* Lysin

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By utilizing conventional techniques of pressure ultrafiltration, gel filtration chromatography, diethylaminoethyl cellulose chromatography, and preparative polyacrylamide electrophoresis, the A component of the group D lysin produced by *Streptococcus zymogenes* has been purified to a state of apparent homogeneity when determined by the techniques of anionic and cationic disc gel electrophoresis. The A component was found to be a protein possessing a molecular weight of 27,000, a sedimentation coefficient approximating 3.2S, and a net negative charge at physiological pH.

Brock and Davie (4) showed that the lysin excreted by a group D streptococcus, *Streptococcus zymogenes* X-14, during logarithmic growth was both bacterioytic and hemolytic. In addition, they presented evidence that the lysin probably achieved bacteriolysis of certain gram-positive organisms by attacking the cell membrane (5). The lytic effect was demonstrated on spheroplasts after removal of membrane-associated teichoic acids. The latter were demonstrated to be inhibitors of the lysin (6).

This laboratory has since isolated mutants of *S. zymogenes* X-14 which demonstrated that the lysin was composed of at least two different macromolecular components (7). The L component appeared to possess the catalytic activity, whereas the A component behaved in the manner of a typical enzyme activator. Hemolytic activity was linearly dependent on the amount of L substance present, whereas activity increased hyperbolically with increasing A substance until a saturation plateau was reached. When the A/L ratio became sufficiently large, activity was inhibited.

We speculated that lysin may serve some in vivo function for the organism in regard to membrane biogenesis, maintenance, or transport. Since the in vitro activity of the lysin could apparently be regulated by varying concentrations of the A component and is subject to control by teichoic acids, it seemed possible that in vivo activity may be responsive to similar regulatory mechanisms. With this in mind, the physical and chemical characterization of these components became of great interest to this laboratory. This report is concerned with the purification and the physical and chemical characterization of the A component.

**MATERIALS AND METHODS**

**Media.** Brain Heart Infusion (BHI) broth (Difco) was used as the growth medium for all strains. These were routinely carried on BHI agar plates containing 5% sheep erythrocytes.

**Buffers.** Phosphate-buffered saline (PBS-A) was used in a concentration of 0.01 M phosphate containing 0.145 M NaCl (pH 6.8) for all hemolytic assays. Phosphate buffer (PBS-B) at the same phosphate concentration and pH but with 0.125 M NaCl was used as the Bio-Gel P-30 elution buffer. PBS-B containing 0 to 0.3 M NaCl was used for the diethylaminoethyl (DEAE) cellulose elution.

**Preparation of rabbit erythrocytes.** Rabbit erythrocytes were obtained by cardiac puncture and suspended in an equal volume of Alsever's solution. Before use, the cells were washed four times with PBS-A and suspended at 2% by volume in the same buffer.

**Preparation of A and L supernatant fluids.** The A supernatant fluid was prepared by adding a 10% inoculum of an overnight culture of an A-producing mutant (A18) into sterile BHI medium and incubating at 37 C with slow shaking. When the culture turbidity reached 150 Klett units (no. 54 filter), the cells were removed by centrifugation at 4 C for 30 min at 20,000 x g. (The yield was approximately 0.2 mg, dry weight, of cells/ml.) The L supernatant fluid was prepared in a similar manner by using an L-producing mutant (L16), except that culture was grown to about 190 Klett units (corresponding to approximately 0.5 mg, dry weight, of cells/ml). The supernatant fluids from each mutant were then refrigerated at 4 C until required for use.

**Chromatography.** Materials. All filtration gels were purchased from Bio-Rad Laboratories. DEAE cellulose with a capacity of 0.90 meq/g was obtained from

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1 Predoctoral trainee of National Science Foundation (grant GZ-914).
Schleicher & Schuell Co. Thin-layer silica gel plates were obtained from Distillation Products Industries.

**Polyacrylamide gel electrophoresis.** Analytical electrophoresis and preparative disc gel electrophoresis were performed by employing 7.5% polyacrylamide gels. By using a Buchler electrophoresis apparatus, anionic (pH 9.3) and cationic (pH 4.3) analytical electrophoretic systems were prepared by the methods provided by Buchler Instruments (9) with some modifications. No stacking gels were employed in either system, and 0.14% potassium persulfate was used as the polymerization catalyst in the anionic system. In each system, the electrophoresis was run at 2.5 mA per gel for about 60 min. After completion of electrophoresis, the gels were fixed in 20 ml of 12.5% trichloroacetic acid (w/v) for 30 min, and then 2.0 ml of 1% Coomassie brilliant blue was added. The gels were stained for 18 hr and destained in 10% trichloroacetic acid (w/v). Preparative electrophoresis was achieved on a Shandon preparative polyacrylamide electrophoresis apparatus by using the same gel and buffering systems as were used in the anionic electrophoresis system.

**Molecular weight determination.** The method of Hedrick and Smith (8), as modified by Lipshitz and Lebowitz (Biophys. Soc. Abstr., 1971, p. 71a; and personal communication), was used for this determination. The method employs polyacrylamide gel electrophoresis of a number of proteins with known molecular weights in the range of 10,000 to 50,000. The mobility of these proteins is measured with respect to the mobility of bromophenol blue in a variety of gel concentrations. The slope of a plot of the relative mobility as a function of gel concentrations gives the "retardation coefficient difference." Lipshitz and Lebowitz have determined a standard curve of "retardation coefficient difference" utilizing 15 different proteins. The method is said to have a 5% error factor.

The "retardation coefficient difference" for A component was determined in a similar manner, and its molecular weight was obtained by comparison with the standard curve.

**Sedimentation coefficient.** The determinations of S_{20,w} values were performed by the procedure of Martin and Ames (13). Five-milliliter linear sucrose gradients were made from 5 to 20% (w/v) in 0.01 M phosphate buffer, pH 7.0. All runs were centrifuged in an SW50L swinging bucket rotor for 12 hr at 39,000 rev/min at 0 C in a model L2-65B Beckman preparative ultracentrifuge. Catalase and hemoglobin were used as markers. Catalase was purchased from Worthington Biochemical Corp., and hemoglobin was kindly provided by Amnon Lipshitz, Syracuse University. In all runs, 0.1 ml of a post-Bio Gel P-30 A component sample containing either 1 mg of catalase of 10 mg of hemoglobin was applied to each gradient. Catalase and hemoglobin were spectrophotometrically determined at 405 and 541 nm, respectively. The A component was detected by the hemolytic assay.

**Concentration procedure.** Concentration of the A component was achieved in all steps by pressure ultrafiltration with an Amicon 65-ml filtration cell and a UM-10 membrane under 60 psi of nitrogen.

**Chemical determinations.** Protein was determined routinely by the method of Kalckar (12). The Allen modified technique of Fiske-SubbaRow-King (1) was used for the assay of hydrolyzable phosphate. The antherone procedure (11) was employed for the detection of hexoses. The method of Bligh and Dyer (3) employing chloroform-methanol was used to extract possible lipids. Iodine vapors and a commercially prepared molybdenum blue spray purchased from Applied Science Laboratories, Inc. were used to detect lipids.

**Amino acid analysis.** The A component sample obtained from preparative electrophoresis was concentrated by pressure ultrafiltration to about 3 ml and desalted on a Bio-Gel P-30 column (36 by 2 cm) equilibrated with double-distilled water. The active fractions were concentrated to approximately 2 ml, lyophilized, and digested in vacuo for 24 hr at 110 C with 1.0 ml of constant-boiling HCl. The hydrolyzed sample was evaporated to dryness and dissolved in 0.5 ml of 0.2 M citrate (pH 2.2) and analyzed on a Beckman-Spinco model 116 amino acid analyzer by the accelerated procedure of Spackman (Fed. Proc. 22:244, 1963) and of Moore, Spackman, and Stein (14).

**Quantitation of A component.** For survey type of analysis, e.g., the assay of column fraction, the standard hemolytic assay previously reported (2, 10) was used. In this case activity is expressed as the reciprocal of the time for initial lysis (T_0) to occur. The reciprocals are multiplied by 10^4 to give convenient numbers. This method of measurement was used in Fig. 1, 2, and 3.

However, assessment of the effectiveness of the purification protocol required a modified approach. The hyperbolic response of hemolytic activity (8) to increasing amounts of A component (in the presence of a constant amount of L component) imposed severe limitations on quantitation of A component. Nevertheless, over a rather restricted range, one can obtain a reasonably linear relationship between activity and amount of A component. This requires using extremely small volumes of A component preparations in the presence of a relatively large and constant volume of L supernatant fluid.

By plotting activity versus volume of A component added and subjecting these data to a linear regression analysis, a reasonably straight line was obtained with a correlation coefficient of at least 0.98 and a standard deviation no greater than 0.002. Since the regression line intersected the origin, the slope could be used as a direct measure of the activity per milliliter in that particular preparation. Specific activity from each preparation was defined as A component units per total milligram of protein.

**RESULTS**

The purification protocol for the A component of the group D lysin and the yields from each step are presented in Table 1. By employing conventional techniques of ultrafiltration, gel filtration, anion exchange chromatography, and preparative disc gel electrophoresis, the A component was purified approximately 384-fold to a state of apparent homogeneity with about a 5% yield of activity.

The experimental procedure first involved concentrating a 150-ml volume of crude culture su-
permatant fluid from mutant A18 approximately 10-fold by pressure ultrafiltration. The sample was filter-sterilized with a 43-mm membrane filter (Millipore Corp., pore size 0.45 μm), loaded onto a Bio-Gel P-30 column (90 by 4 cm) equilibrated with PBS-B, and eluted with the same buffer at a flow rate of 50 ml/hr. Figure 1 represents the activity and protein profiles from this step.

The peak activity fractions (indicated by the bar in Fig. 1) were pooled and concentrated to approximately 20 ml. The salt concentration in this sample was adjusted to less than 0.05 M by the addition of an appropriate volume of cold phosphate buffer. The sample was then applied to a DEAE cellulose column (36 by 2 cm) equilibrated with 0.01 M phosphate buffer, pH 7.0. The sample was then stepwise eluted with 250-ml volumes of phosphate buffer containing 0.0, 0.10, 0.15, 0.20, and 0.30 M NaCl in succession. Figure 2 represents the typical activity and protein profiles observed under these conditions.

The peak activity fractions (indicated by the bar in Fig. 2) were pooled and concentrated to about 10 ml, to which was added a sufficient volume of cold distilled water to decrease the salt concentration to less than 0.01 M.

This preparation was then concentrated by pressure dialysis to a volume of 2 ml, resuspended in 20% sucrose (w/v), and placed onto a Shandon preparative electrophoresis apparatus containing 7.5% polyacrylamide gel. Electrophoresis was conducted at 65 ma at a flow rate of 22 ml/hr; fraction size was 1.3 ml. The peak activity fractions from this step were pooled, concentrated to about 2 ml, and examined for homogeneity.

The techniques of anionic (pH 9.3) and cationic (pH 4.3) analytical disc gel electrophoresis (8) were employed to establish homogeneity. In the anionic system, 0.2-ml samples in 20% sucrose (w/v) were applied to 7.5% polyacrylamide...
gels. After electrophoresis, staining, and destaining, a single protein band was observed.

A duplicate gel, which was not subjected to trichloroacetic acid fixation and Coomassie brilliant blue staining, was employed to determine the location of A component activity. The gel was sectioned into 0.5-cm pieces, macerated with a spatula, and suspended in 1 ml of PBS-A. After 30 min of incubation at 37°C, a 0.2-ml sample was removed from each fraction and assayed for A component activity. Activity was found only in the section corresponding to the location of the observed protein band.

The cationic electrophoresis was performed with the same volume of sample and, upon destaining, one protein band was observed. No activity could be detected in a duplicate unstained gel, suggesting that the A component molecule was denatured at the lower pH.

Since only one protein band was detected in both the anionic and cationic electrophoretic systems, the post-preparative electrophoresis sample was considered homogeneous by these criteria. Isoelectric focusing was attempted numerous times but, due to technical difficulties in protein staining, reliable results could not be attained.

The method of Liphshitz and Lebowitz was employed to determine the molecular weight of the A component from various stages of the purification. Post-Bio-Gel P-30, post-DEAE cellulose, and post-preparative disc gel electrophoresis samples were subjected to this analysis. Since the post-Bio-Gel P-30 and post-DEAE cellulose samples contained heterogeneous protein, duplicate gels were run for each to determine which protein band corresponded to A component activity. With this technique, the molecular weight of the A component was determined to be 27,000 regardless of the preparation studied.

The method of Martin and Ames (13), using linear sucrose gradients, was employed to estimate the sedimentation coefficient of the A component. When hemoglobin (4.3S) was used as the marker protein, the activator was found to have a sedimentation coefficient of 3.2S as represented in Fig. 3B. When catalase (11.3S) was used as a marker protein, an estimated sedimentation coefficient of about 3.0S was obtained as shown in Fig. 3A.

A variety of chemical analyses were also made on the purified preparations. The Allen modified Fiske-SubbaRow-King (1) procedure was used to assay for hydrolyzable organic phosphate. The chloroform-methanol procedure of Bligh and Dyer (3) was used to extract lipids. After extraction, the chloroform layer was concentrated under nitrogen and spotted on silica gel thin-layer plates. The plates were chromatographed for 5 hr with diisobutylketone-acetic acid-water (8:5:1, v/v), air-dried for 12 hr, and exposed to iodine vapors for 15 min to detect lipids. The chromatograms were then sprayed with molybdenum blue reagent to detect phospholipids. The anthrone test (11) was employed to determine the presence of hexoses. In each case, test samples contained slightly more than 100 µg of pro-

![Fig. 2. DEAE-cellulose chromatography of A component. The sample was stepwise eluted with 250-ml volumes of 0.01 M phosphate buffer (pH 7.0) containing 0.0, 0.10, 0.15, 0.20, and 0.30 M sodium chloride in succession. Fractions (8 ml) were collected. Activity is expressed as the reciprocal of time to give initial lysis, T₀. The reciprocals are multiplied by 10⁴ for convenient numbers. Bar indicates fractions pooled for subsequent step. Symbols: ●, protein; ○, hemolytic activity.](http://jb.asm.org/DownloadedFrom)
tein. In no case was there evidence of any of these materials. In our hands the Allen procedure will detect less than 1 μg of phosphate, the molybdenum blue spray reagent less than 2 μg of phosphate, the anthrone test approximately 10 μg of glucose, and the iodine vapors approximately 2 μg of lipid (as either phosphatides or fatty acids). Thus, within these limits, the A component contained no lipid, phospholipid, or carbohydrate.

An amino acid analysis was made on four separate preparations. Table 2 represents the relative amino acid composition based on a molecular weight of 27,000. The experimentally determined number of micromoles of aspartic acid was arbitrarily set as equal to 10 residues, and all other amino acids were made relative to aspartic acid by dividing the number of micromoles of each by the number of micromoles of aspartic acid. Column A gives the results of this operation. The arbitrary residue numbers were then multiplied by the respective molecular weights of each amino acid to give the relative contribution of each to the molecular weight of the protein. These values are shown in column B. The sum of the column B values was then divided into 27,000 to obtain the factor required to convert the arbitrary residue numbers in column A into the residue values necessary to sum up to a molecular weight of 27,000. These corrected residue values are given in column C and have been rounded off to the nearest whole number.

It should be observed that acidic amino acids (glutamic acid and aspartic acid) predominate

![Graph A](image1.png)  ![Graph B](image2.png)

**Fig. 3.** Zonal centrifugation of A component with catalase (A) and hemoglobin (B) as markers on 5-ml linear sucrose gradients (5 to 20%). Fraction size was six drops. Activity is expressed as the reciprocal of the time to initial lysis, Tₐ. The reciprocals are multiplied by 10² for convenient numbers. Symbols: catalase, △; hemoglobin, ○; catalase, △.
P-30 desalting step was not 100% efficient in removing the glycine-tris(hydroxymethyl)amino-methane buffer used during the preparative electrophoresis step.

DISCUSSION

By employing conventional techniques of ultrafiltration, gel filtration, anion exchange chromatography, and preparative disc gel electrophoresis, the A component of the group D lysin has been purified to a state of homogeneity as established by the criteria of anionic and cationic disc gel electrophoresis. Chemical analysis of the purified substance indicates that it is a protein having no phosphate, hexose, or lipid materials associated with it. The amino acid analysis results suggest that the molecule would possess an overall net negative charge at physiological pH. This result was further substantiated by the fact that the molecule binds to DEAE cellulose. The molecule has an estimated sedimentation coefficient of 3.25 and a molecular weight of 27,000. This latter result is curious in view of the fact that the activity in crude culture supernatant fluids is excluded by Bio-Gel P100 and only barely included by P200 (7). Perhaps the A component can exist in an aggregated state or conceivably it is associated with another substance in crude preparations.

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LITERATURE CITED