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Staphylococcal β-Hemolysin

I. Purification of β-Hemolysin[1]

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The purification of staphylococcal β-hemolysin was accomplished by the successive use of three protein fractionation methods. The first method employed was a double precipitation with the use of ammonium sulfate at 65% saturation. The second phase of purification used Sephadex G-100 column fractionation. The third phase utilized either carboxymethyl cellulose or diethylaminoethyl cellulose fractionation. The last two fractionation methods both resulted in the separation of a relatively high concentration of cationic hot-cold lysin and a low concentration of anionic hot-cold lysin. Because of the low concentration of the anionic component, its purity could not be assessed. However, the purity of the cationic component was demonstrated by immunodiffusion, microimmunoelectrophoresis, and by disc polyacrylamide gel electrophoresis. In addition, antisera against purified cationic β-hemolysin yielded one line of precipitate when tested against the original crude β-hemolysin. The purified cationic β-hemolysin was stable in the lyophilized state. Crude β-hemolysin was dermonecrotic, whereas purified cationic β-hemolysin was not dermonecrotic even after Mg^{++} activation.

The existence of β-hemolysin as a distinct product of *Staphylococcus aureus* has been generally accepted for several years (1, 8, 23). More recently, staphylococcal β-hemolysin has been partially purified by G. V. Keefer (Ph.D. Thesis, Univ. of Wisconsin, Madison, 1964), Wiseman (24), Doery et al. (5), Chesbro et al. (2), and Jackson (11). R. Haque (Ph.D. Thesis, Ohio State Univ., Columbus, 1963) described a procedure for the purification of β-hemolysin which involved the use of acetone precipitation followed by diethylaminoethyl (DEAE) cellulose column fractionation. He reported the separation of two hot-cold lysins which he referred to as cationic and anionic β-hemolysin. He also reported that the cationic hemolysin was immunoelectrophoretically pure.

The work reported herein confirms the work of Haque in that cationic and anionic hot-cold lysins were separated. In addition, this report describes a three-step method for purifying cationic β-hemolysin. The purity of the cationic β-hemolysin is demonstrated by four distinct methods.

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MATERIALS AND METHODS

The methods used for the measurement of α- and δ-hemolysin and β-antihemolysin were identical to those employed by Manohar et al. (15).

Measurement of β-hemolytic activity. Sheep erythrocytes were washed three times and resuspended in phosphate-buffered saline (PBS) made up of 0.145 M NaCl, 0.01 M phosphate buffer, *p*H 7.2, and containing 0.002 M $MgSO_4$. β-Hemolytic activity was measured by mixing 0.5 ml of a 1% suspension of the sheep erythrocytes with 0.5 ml of serial twofold dilutions of hemolysin. These mixtures were incubated for 1 hr at 37 C followed by 1 hr at 4 C. β-Hemolytic activity was expressed in hemolytic units according to the method of Cooper et al. (3).

Specific activity of β-hemolysin. The number of hemolytic units in 1.0 ml of the sample was divided by the milligrams of protein present in 1.0 ml of the sample. Thus, specific activity was expressed as units per milligram.

Measurement of protein. Protein concentration was measured by the method of Miller (16).

Preparation of crude β-hemolysin. S. aureus strain T19 was used for the production of β-hemolysin. The toxin was produced by the method of K. L. Smith (M.S. Thesis, Univ. of Minnesota, St. Paul, 1963). This method employed the medium of Leonard and Holm (13), containing 1.5% agar. The cultures were incubated for 48 hr at 42 C in an atmosphere containing 50% CO_2 and 50% O_2. The crude toxin was extracted by the method of Tasman

and van der Slot (22). The cells were removed from the extracted hemolysin by centrifugation at 10,000 × *g* for 30 min. The hemolysin was concentrated four fold by pervaporation.

Ammonium sulfate precipitation. An adaptation of the method of Dixon (4) was used to precipitate β-hemolysin. Crude hemolysin was dialyzed overnight against 5 volumes of distilled water at 4 C. The hemolysin was then adjusted to a *p*H of 8. Ammonium sulfate (50.5 g, 65% saturation) was added to 100 ml of hemolysin while the mixture was stirred at room temperature. After 30 min, the precipitate was removed by centrifugation at 15,000 × *g* for 10 min. The precipitate was dissolved in 0.145 M NaCl and dialyzed overnight in the cold against distilled water. This product was again precipitated with ammonium sulfate (65% saturation). The precipitate was removed, dissolved in saline, and dialyzed as before.

Sephadex G-100 column fractionation. Sephadex G-100 (lot no. T02946; particle size, 40 to 120 μ; U.S. Standard; Pharmacia, Uppsala, Sweden) was suspended in water for 2 days. The fine particles were removed with the supernatant fluid, and the slurry was washed five times in PBS. The washed Sephadex G-100 was packed in a column which measured 80 by 3.2 cm. The void volume and the uniformity of packing were determined by use of 0.01% blue dextran in the above buffer. A 5-ml sample (0.8 mg of protein/ml) of ammonium sulfate-precipitated toxin was fractionated at room temperature. The flow rate was adjusted to 11 ml/hr, and 8-ml eluates were collected. Each eluate was analyzed for protein and for α-, δ-, and β-hemolytic activity. The most active β-hemolytic fractions were pooled.

DEAE cellulose column fractionation. The DEAE cellulose used was Whatman-Chromomedia with an exchange capacity of 1.0 meq/g. A column, 57 by 1.8 cm, was prepared by the method of Peterson and Sober (19). The column was flushed with 0.005 M phosphate buffer (*p*H 7.2). A sample which consisted of a pool of the most active eluates from Sephadex fractionation was dialyzed for 18 hr in the cold against the above buffer. A 10-ml amount of the sample (162 μg of protein/ml) was fractionated at a flow rate of 6 ml/hr at 4 C. Linear gradient elution was accomplished by use of 0.6 M NaCl. The 4-ml eluates collected were analyzed for β-hemolytic activity and for protein content.

Carboxymethyl (CM) cellulose column chromatography. The CM cellulose used was obtained from Schleicher & Schuell Co., Keene, N.H., and had an exchange capacity of 0.72 meq/g. The method of Peterson and Sober (19) was used to prepare the column. The column (57 by 1.8 cm) was flushed with 0.005 M phosphate buffer (*p*H 7.0) at 4 C. A sample of the pool obtained from Sephadex fractionation was dialyzed against the above buffer. A 10-ml sample (162 μg of protein/ml) was fractionated at 4 C by use of a flow rate of 6 ml/hr. Linear gradient elution was accomplished by use of 0.2 M phosphate buffer (*p*H 7.5) containing 3% NaCl. The 4-ml eluates were analyzed for β-hemolytic activity and for protein content.

Rabbits. Adult New Zealand white rabbits were used throughout these experiments. They were free from antibodies to staphylococcal products as demonstrated by immunoelectrophoretic tests of their serum against crude β-hemolysin.

Preparation of crude anti-β-hemolysin. Rabbits were immunized with crude β-hemolysin by use of Freund Incomplete Adjuvant (Difco). The procedure used was that of Freund (7). The rabbit antiserum used in these studies contained 1,024 units of anti-β-hemolysin. It was stored at −20 C.

Preparation of antisera against cationic and anionic β-hemolysins. Four rabbits were immunized with anionic β-hemolysin and four with cationic β-hemolysin. The procedure used was that of Freund (7). The antisera were stored at −20 C.

Microimmunoelectrophoresis. Schiedeger's (21) method was used with modification. Noble agar, 0.8% (Difco), was used in phosphate borate buffer (*p*H 8.2; ionic strength, 0.05). The antigen was allowed to migrate for 2 hr at a potential of 4 v/cm of gel length. After electrophoresis of the antigen, antiserum was added, and the slides were incubated for 48 hr at room temperature in a moist chamber. The slides were washed for 5 days with sterile saline and stained with amido black (10B-color index, 246).

Immunodiffusion. The method of Ouchterlony (18) was used with slight modification. Veronal buffer (0.05 M, *p*H 8.2) containing a 1:10,000 concentration of Merthiolate and 0.8% Noble agar was used. The method of Korngold and Lipari (12) was used to prepare the wells. In all cases, 0.5 ml of the antigen from various steps in the purification procedure and 0.5 ml of crude antisera were used. The plates were incubated for 72 hr at room temperature in a moist chamber. The plates were then washed for 5 days with sterile saline and stained with amido black (10B-color index, 246).

Immunoelectrophoresis. The method of Grabar (9) was used with one modification. Phosphate borate buffer (*p*H 8.2; ionic strength, 0.05) was used instead of Veronal buffer.

Disc polyacrylamide gel electrophoresis. The procedure used was similar to that of Reisfeld et al. (20). It differed in that it employed 15% instead of 7.5% acrylamide in the small-pore gel.

RESULTS

Purification of β-hemolysin. The procedure used for the purification of β-hemolysin consisted of three successive steps: (i) double precipitation with ammonium sulfate; (ii) Sephadex G-100 column fractionation; (iii) column fractionation with either CM cellulose or DEAE cellulose

These studies revealed that a double precipitation with ammonium sulfate at 65% saturation (*p*H 8.0) resulted in the recovery of almost all of the β-hemolysin. The first precipitation resulted in a 10-fold increase in specific activity, whereas

reprecipitation with ammonium sulfate (65% saturation) resulted in a 23-fold increase in specific activity (Table 1).

Fractionation with Sephadex G-100 resulted in a 46-fold increase in specific activity of the β-hemolysin. The elution profile shown in Fig. 1 revealed four protein peaks, only one of which showed β-hemolytic activity. None of the eluates showed α- or δ-hemolytic activity. Recycling of this active material through Sephadex G-100 resulted in a very slight increase in specific activity. Therefore, only one Sephadex fractionation was used in the purification procedure.

Additional fractionation of the above product by use of DEAE cellulose column chromatography yielded two β-hemolytic peaks (Fig. 2). The first β-hemolytic peak was eluted with the starting buffer, and contained a relatively high concentration [128 hemolytic units (HU)] of hemolysin. This hot-cold lysin was designated cationic β-hemolysin. The second β-hemolytic peak appeared after linear gradient elution with 0.6 M NaCl. This peak showed a relatively low concentration (10 HU) of hemolysin. This hot-cold lysin was designated anionic β-hemolysin. The cationic β-hemolysin was purified 230-fold by this procedure and had a specific activity of 64,000 units/mg.

TABLE 1. *Specific activity obtained by the various steps of the purification procedure*

Purification step	HU/ml	Protein (mg/ml)	Specific activity	Purification (-fold)
Crude hemolysin	5,340	19.2	278	1
Twice $(NH_4)_2SO_4$	5,200	0.80	6,500	23
Sephadex G-100	2,048	0.162	12,800	46
DEAE cellulose	256	0.004	64,000	230
CM cellulose	614	0.009	68,200	245

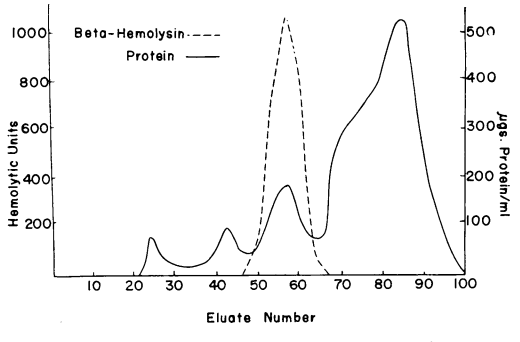

FIG. 1. *Fractionation of ammonium sulfate-precipitated β-hemolysin by use of Sephadex G-100. Buffer, phosphate-buffered saline (pH 7.2); flow rate, 11 ml/hr; eluate volume, 8 ml.*

β-Hemolysin obtained from Sephadex fractionation was also purified by use of CM cellulose column chromatography. This procedure also yielded two β-hemolytic peaks (Fig. 3). The anionic β-hemolysin was eluted with the starting buffer and showed a peak activity of 18 HU. The cationic β-hemolytic peak appeared after linear gradient elution with 0.2 M phosphate (*p*H 7.5) containing 3% NaCl, and showed a peak activity of 312 HU. The cationic β-hemolysin was purified 245-fold by this procedure and had a specific activity of 68,200 units/mg.

Since the anionic β-hemolysin was not detect-

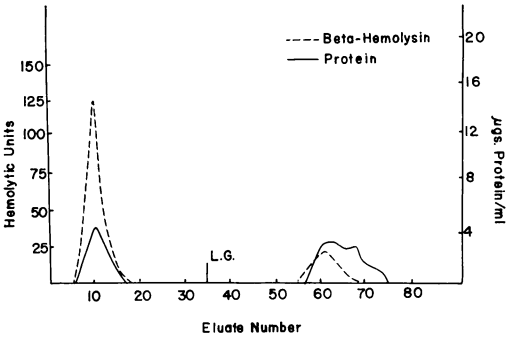

FIG. 2. *Fractionation of β-hemolysin by use of DEAE cellulose. The β-hemolysin was a pool of the active eluates obtained from Sephadex fractionation. Exchange capacity, 1.0 meq/g; column size, 57 by 1.8 cm; flow rate, 6 ml/hr; eluate volume, 4 ml; starting buffer, 0.005 M phosphate (pH 7.2); linear gradient (L.G.) elution with the use of 0.6 M NaCl started after the 35th eluate.*

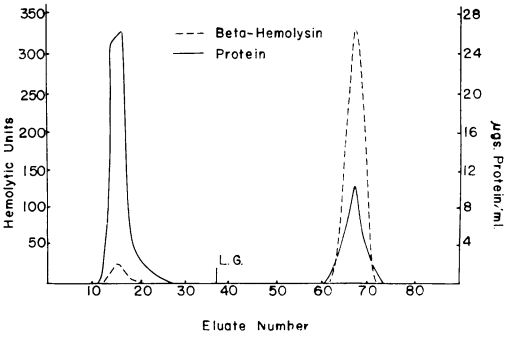

FIG. 3. *Fractionation of β-hemolysin by use of CM cellulose. The β-hemolysin was a pool of the active eluates obtained from Sephadex fractionation. Exchange capacity, 0.72 meq/g; column size, 57 by 1.8 cm; flow rate, 6 ml/hr; eluate volume, 4 ml; starting buffer, 0.005 M phosphate (pH 7.0); linear gradient (L.G.) with the use of 0.2 M phosphate buffer (pH 7.5) containing 3% NaCl started after the 38th eluate.*

able by immunodiffusion, immunoelectrophoresis, and disc polyacrylamide gel electrophoresis, proof of purity of this component was not possible. Therefore, the remainder of this study dealt with the cationic β-hemolysin.

Purity of β-hemolysin. Several methods were used to test the purity of the final cationic β-hemolysin. These methods were immunodiffusion, microimmunoelectrophoresis, and disc polyacrylamide gel electrophoresis. In addition, antiserum produced in rabbits against purified β-hemolysin was tested against crude β-hemolysin in an attempt to detect trace antigenic impurities in the purified toxin.

Crude β-hemolysin and hemolysin from the first two steps of purification were concentrated by lyophilization and reconstituted to approximately equivalent levels of protein. These antigens were then subjected to immunodiffusion analysis against crude anti-β-hemolysin. It may be seen from Fig. 4 that crude β-hemolysin showed several lines of precipitation, ammonium sulfate-fractionated β-hemolysin yielded fewer lines, and hemolysin fractionated on Sephadex gave only two lines of precipitation.

Figure 5 shows the results of immunodiffusion analysis with the use of crude β-hemolysin and CM cellulose-fractionated hemolysin against crude anti-β-hemolysin. Crude β-hemolysin showed several lines of precipitate, whereas the purified β-hemolysin showed one line of precipitate. DEAE-fractionated β-hemolysin also produced one line of precipitate when tested against crude anti-β-hemolysin by immunodiffusion methods.

Figure 6 shows the results of microimmunoelectrophoresis with the use of crude β-hemolysin and purified cationic β-hemolysin against crude anti-β-hemolysin. Crude β-hemolysin yielded at least six components, whereas purified hemolysin again showed just one line of precipitate, which was located on the cathode side of the slide. DEAE-fractionated β-hemolysin showed the same results.

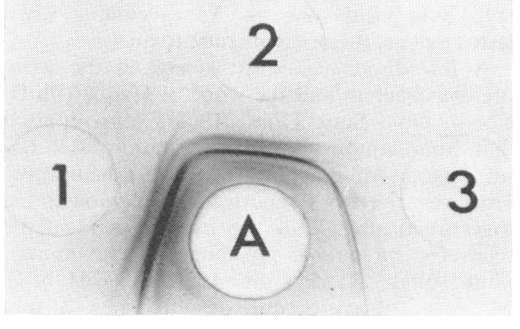

FIG. 4. *Immunodiffusion analysis of the products of ammonium sulfate precipitation and Sephadex G-100 column fractionation. (A) Antiserum against crude β-hemolysin. (1) Crude β-hemolysin. (2) Product of double ammonium sulfate precipitation. (3) Product obtained after ammonium sulfate precipitation and Sephadex G-100 fractionation. Protein content of wells 1, 2, and 3 equals 1 mg/ml.*

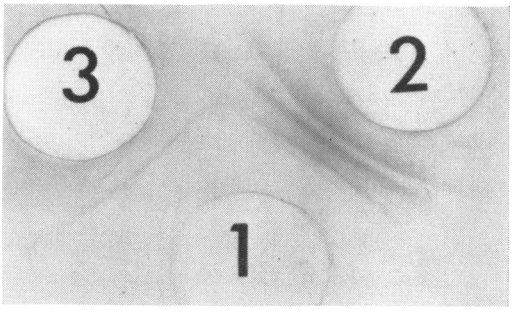

FIG. 5. *Immunodiffusion analysis comparing crude β-hemolysin and purified β-hemolysin. (1) Antiserum against crude β-hemolysin. (2) Crude β-hemolysin. (3) Purified cationic β-hemolysin. Protein content of wells 2 and 3 equals 50 μg/ml.*

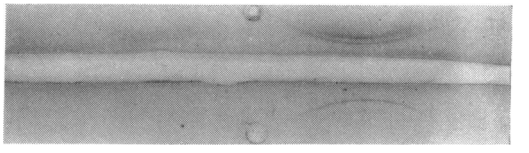

FIG. 6. *Microimmunoelectrophoretic analysis comparing crude β-hemolysin and purified β-hemolysin. Upper well, crude β-hemolysin. Lower well, purified cationic β-hemolysin. Central moat, antiserum against crude β-hemolysin.*

Disc polyacrylamide gel electrophoresis (Fig. 7) with the use of crude β-hemolysin showed several bands of protein. Purified cationic β-hemolysin showed only one band, which migrated to the cathode. Again, DEAE fractionation yielded the same result.

Immunoelectrophoretic studies were conducted with antiserum produced against purified β-hemolysin. This technique was utilized because Heidelberger et al. (10) demonstrated that extremely small amounts of antigen may call forth the production of relatively large amounts of antibody. It was, therefore, decided to attempt to stimulate the production of antibodies to possible trace antigenic impurities in the purified preparation. These studies revealed (Fig. 8) that crude β-hemolysin showed several lines of precipitate with crude anti β-hemolysin, whereas only one line of precipitation appeared when antibodies against purified cationic β-hemolysin were used.

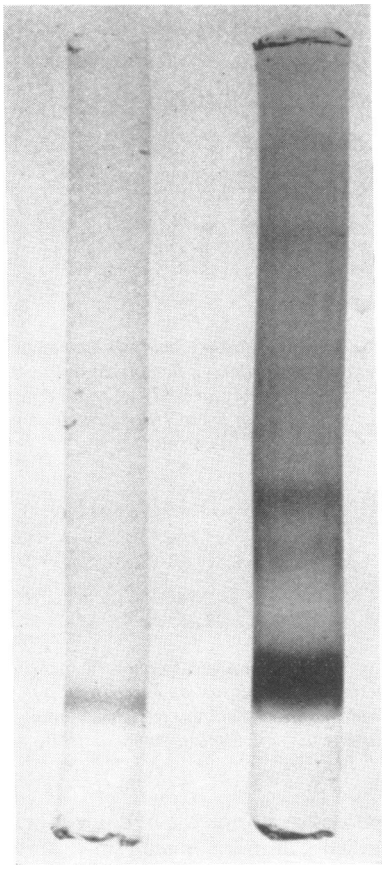

FIG. 7. *Disc polyacrylamide gel electrophoretic analysis of crude β-hemolysin and purified β-hemolysin. Left, purified cationic β-hemolysin (50 μg of protein). Right, crude β-hemolysin (180 μg of protein).*

Additional studies with antiserum prepared against purified cationic β-hemolysin revealed that this monovalent serum inhibited cationic and anionic β-hemolysin to approximately the same degree.

Purified cationic β-hemolysin was unstable at room temperature. When stored at 4 C, it was active for about 3 days. However, when it was lyophilized and stored in a desiccator at 4 C, it was stable for as long as 4 months. Crude β-hemolysin was dermonecrotic to rabbits, but neither the purified cationic β-hemolysin nor the anionic β-hemolysin was dermonecrotic even after Mg^{++} activation.

DISCUSSION

The three-step procedure for the purification of staphylococcal β-hemolysin presented herein yielded a final product with a specific activity of approximately 68,000 units/mg. The third step

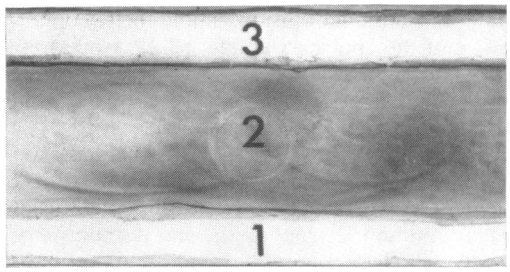

FIG. 8. *Immunoelectrophoretic comparison of antiserum prepared against purified cationic β-hemolysin and antiserum against crude β-hemolysin. (1) Antiserum against crude β-hemolysin. (2) Crude β-hemolysin (4.3 mg of protein). (3) Antiserum against purified cationic hemolysin.*

of this procedure employed either CM cellulose or DEAE cellulose fractionation. Both of these methods of fractionation resulted in the separation of a cationic and an anionic hot-cold lysin. In both cases, the anionic hot-cold lysin was found in low concentration, accounting for 5 to 10% of the hot-cold lytic activity. These two serologically related hot-cold lysins could have been present in the original crude hemolysin, or one of these lysins could be an altered form of the other. Such alteration may have been the result of phenomena such as conjugation or dissociation. However, the information available at present does not allow a definitive conclusion.

The purity of the major hot-cold lysin (cationic) has been demonstrated by immunodiffusion, microimmunoelectrophoresis, and disc polyacrylamide gel electrophoresis. In addition, immunoelectrophoretic studies have demonstrated that antibody produced against purified cationic hot-cold lysin yields one line of precipitate when tested against the original crude toxin.

A few discrepancies exist between the work presented herein and the work of Haque (Ph.D. Thesis, Ohio State Univ., 1963), who reported that ammonium sulfate precipitation was unsatisfactory for the purification of β-hemolysin. However, the work reported here demonstrated that practically all of the β-hemolysin can be recovered by double precipitation with ammonium sulfate at 65% saturation at a *p*H of 8. Moreover, this procedure resulted in a 23-fold increase in specific activity. Another discrepancy between this report and the work of Haque involved the elution of β-hemolysin from CM cellulose. He felt that the use of CM cellulose was inadvisable since it was difficult to elute β-hemolysin from the column. However, this report demonstrated that almost all of the β-hemolysin can be eluted from CM cellulose by use of a linear

gradient elution with 0.2 M phosphate (*p*H 7.5) containing 3% NaCl.

This work confirms the work of Haque with regard to the separation of a cationic and an anionic hot-cold lysin. However, the anionic component isolated in this study did not lyse rabbit erythrocytes, whereas Haque reported that the anionic hot-cold lysin lysed rabbit erythrocytes. These discrepancies probably cannot be resolved at the present time, since the two studies employed different cultural methods for the production of β-hemolysin, different strains of *S. aureus*, and different methods of fractionation.

Chesbro et al. (2) demonstrated that β-hemolysin is a cationic molecule. Haque reported that his major hot-cold lysin was cationic. This work also showed that the major hot-cold lysin had cationic properties. From these results, it seems probable that the classical β-hemolysin is a cationic compound.

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