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PURITY OF STAPHYLOCOCCAL δ -HAEMOLYSIN OBTAINED BY THREE DIFFERENT PROCEDURES

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TO STUDY the biological, physical, and chemical characteristics of staphylococcal δ -haemolysin, and also to determine its role in host-parasite interactions we needed to obtain a purified preparation of δ -haemolysin. At present, three methods (Wiseman and Caird, 1968; Kreger, Zabovetzky and Bernheimer, 1971; Kantor, Temples and Shaw, 1972) have been reported for purifying δ -haemolysin. In this investigation we tried to compare these methods and to test the purity of the δ -haemolysin preparations so obtained. Our findings indicate that when biological tests were used for establishing the purity of the final products none of the methods tested yielded a pure preparation of δ -haemolysin.

MATERIALS AND METHODS

Strain. The 146P strain of *Staphylococcus aureus* previously described (Murphy and Haque, 1971) was used.

Crude δ -toxin. This was obtained by growing the culture on dialysis membranes as previously described (Murphy and Haque, 1971).

Identification of haemolysins. The various haemolysins were identified by the electrophoretic localisation technique (Haque, 1967). δ -Haemolysin was defined as the haemolytic moiety that upon electrophoresis in a 1% gel of Ion Agar no. 2 (Oxoid) in barbital buffer (pH 8.4) at 5 mA per cm for 1½ h, remained at the origin and lysed human, rabbit and horse erythrocytes. α -Haemolysin under these same conditions migrated 18–20 mm toward the cathode and lysed rabbit, sheep and horse erythrocytes, and in concentrated preparations it lysed human erythrocytes. β -Haemolysin migrated approximately 36 mm toward the cathode and lysed sheep erythrocytes only.

Measurement of δ -haemolysin. The tube titration procedure as previously described (Murphy and Haque, 1971) was used. The haemolytic titre was the highest dilution of a haemolytic sample that gave 50% lysis of a 1% suspension of washed human erythrocytes and the titre represented the number of haemolytic units (HU) in the sample. Specific activity of

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δ -haemolysin was determined by dividing the haemolytic titre by the protein concentration (mg per ml). Protein concentration was determined by the formula: Protein (mg per ml) = $1.45 \text{ OD}_{280} - 0.74 \text{ OD}_{260}$ (KalcKav, 1947), where OD is the optical density at the stated wavelength (nm).

Determination of other biologically active products. The crude and the purified preparations of δ -haemolysin were tested for the presence of lipase, egg-yolk factor, esterase, lysozyme, caseinase, deoxyribonuclease, penicillinase, and phosphatase by the agar plate tests previously described (Baman and Haque, 1970). Staphylokinase was detected by incorporating 0.15% fibrinogen (Bovine Fraction No. 1; Nutritional Biochemicals Corporation, NBC) and 0.03% thrombin (NBC) in a 1% solution of sterile molten (45°C) agarose in 0.001M phosphate buffer, pH 7.2, containing 0.02% NaN_3 and 10 mM MgCl_2 . For testing, the samples were placed in wells in the agarose and the plate was incubated at 37°C overnight. Presence of staphylokinase in the samples was indicated by the production of sharply defined zones of clearance on the fibrinogen-thrombin plate. Hyaluronidase was detected by incorporating 0.1% umbilical potassium hyaluronate (NBC) in a 1% solution of sterile molten (45°C) agarose in 0.05M Tris buffer, pH 8.8, containing 0.02% NaN_3 . For testing, samples were placed in wells in the agarose and the plate was incubated at 37°C overnight. The plate was then flooded with a solution of cetyl-trimethyl ammonium bromide. Samples containing hyaluronidase yielded clear zones in an otherwise opaque plate.

Purification procedures. The methods of purification as described by Wiseman and Caird (1968), Kreger *et al.* (1971) and Kantor *et al.* (1972), were closely followed.

Purity tests. The purity of the various preparations of δ -haemolysin was tested by (a) determining the presence of biological activities known to be due to products other than the δ -haemolysin, and (b) the electrophoretic localisation technique.

TABLE I

*The biological and haemolytic activities demonstrable in fractions obtained from crude preparations of staphylococcal δ -haemolysin obtained by the method of Kreger *et al.* (1971)*

Fraction	Size (mm) of zone of the stated activity														Haemolysins detected electrophoretically
	Lipase	Egg-yolk factor	Esterase	Staphylokinase	Lysozyme	Caseinase	Deoxyribonuclease	Penicillinase	Phosphatase	Hyaluronidase	Lysis of human RBC	Lysis of horse RBC	Lysis of rabbit RBC	Lysis of sheep RBC	
1. Crude filtrate	20	18	22	15	10	10	20	14	18	14	15	14	18	13	α δ
2. Supernate after absorption with hydroxylapatite	8	7	14	—	—	6	5	10	10	—	8	9	9	8	δ
3. Eluate from hydroxylapatite with 0.01M potassium-phosphate buffer	6	5	15	10	—	9	4	10	4	8	6	6	7	5	α
4. Eluate from hydroxylapatite with 0.4M potassium-phosphate buffer after 0.01M potassium-phosphate buffer	15	10	13	14	8	7	18	8	—	13	8	10	16	13	δ
5. Eluate from hydroxylapatite with 1.0M potassium-phosphate buffer after 0.4M potassium phosphate buffer (Kreger's final soluble δ -haemolysin)	14	12	12	—	—	—	10	9	8	9	15	10	13	4	δ

— = No zone.

RESULTS

The crude preparation of δ -haemolysin contained lipase, egg-yolk factor, caseinase, deoxyribonuclease, penicillinase, phosphatase, esterase, staphylokinase, lysozyme, and hyaluronidase activities. It had a haemolytic titre of 256 against human erythrocytes, and a protein concentration of 25 mg per ml. It also contained both α - and δ -haemolysins when tested by the electrophoretic localisation technique (Haque, 1967). Expressed in terms of specific activity of δ -haemolysin, the crude preparation represented 10 HU per mg of protein.

When purified by the method of Kreger *et al.* (1971), the final preparation of δ -haemolysin contained lipase, egg-yolk factor, esterase, deoxyribonuclease, penicillinase, phosphatase, hyaluronidase and δ -haemolysin (table I). The hydroxylapatite did not preferentially adsorb δ -haemolysin from the crude filtrate. It adsorbed both α - and δ -haemolysins along with small quantities of other enzymes. However, α -haemolysin was eluted with 0.4M buffer whilst δ -haemolysin was eluted with 1.0M buffer. Both α - and δ -haemolysins so obtained also contained various other enzymes. The final product obtained by this method contained 3 mg protein per ml, and had a haemolytic titre of 64 against human erythrocytes. In terms of specific activity the preparation had 21 HU per mg of protein.

Purification of δ -haemolysin by the method of Kantor *et al.* (1972) yielded a final product that contained lipase, egg-yolk factor, esterase, staphylokinase,

TABLE II

*The biological and haemolytic activities demonstrable in fractions obtained from crude preparations of staphylococcal δ -haemolysin obtained by the method of Kantor *et al.* (1972)*

Fraction	Size (mm) of zone of the stated activity														Haemolysins detected electrophoretically
	Lipase	Egg-yolk factor	Esterase	Staphylokinase	Lysozyme	Caseinase	Deoxyribonuclease	Penicillinase	Phosphatase	Hyaluronidase	Lysis of human RBC	Lysis of horse RBC	Lysis of rabbit RBC	Lysis of sheep RBC	
1. Crude filtrate	20	18	22	15	10	10	20	14	18	14	15	14	18	13	α δ
2. Supernate after absorption with aluminium-hydroxide gel	5	4	—	—	—	—	5	14	—	—	—	—	4	—	α (weak) δ
3. Eluate from aluminium-hydroxide gel with 0.08M potassium phosphate buffer	14	13	16	14	4	9	15	12	4	14	7	6	18	9	α δ
4. Eluate from aluminium-hydroxide gel with 0.5M potassium-phosphate buffer after 0.08M potassium-phosphate buffer (Kantor's final δ -haemolysin)	18	18	20	14	10	5	20	14	—	14	14	12	18	8	α δ

— = No zone.

lysozyme, caseinase, deoxyribonuclease, penicillinase, and hyaluronidase activities as well as α - and δ -haemolysins (table II). The aluminium-hydroxide gel (alumina) used in this purification scheme did not preferentially adsorb the δ -haemolysin. The final product obtained by this method contained 8 mg protein per ml, and had a haemolytic titre of 512. This represents a specific activity of 64 HU per mg of protein.

The final preparation of " δ -haemolysin" obtained by the method of Wiseman and Caird (1968) did not contain δ -haemolysin, but it contained α -haemolysin, deoxyribonuclease, penicillinase and phosphatase activities (table III). The initial step in this fractionation procedure consisted of mixing the crude preparation of δ -haemolysin with acetate buffer, pH 4.0. This yielded a precipitate containing both the α - and δ -haemolysins. Further processing of this precipitate consisted of dissolving it in Tris buffer, pH 9.0, dialysis against phosphate buffer, pH 7.0, centrifugation and then adsorption on to hydroxylapatite. The α - and δ -haemolysins, as with the method of Kreger *et al.* (1971), were not completely adsorbed on hydroxylapatite; some of these haemolysins remained in the supernate. The amounts of α - and δ -haemolysins that were adsorbed on hydroxylapatite were eluted with 2M NaCl and then applied to DEAE-cellulose. Here the α -haemolysin appeared in the void volume whilst the δ -haemolysin was adsorbed by the ion exchanger and could not be detected in the fractions obtained by developing the column with 0.1 and 0.5M phosphate buffer. The final preparation (5a, table III) obtained by this method had 0.5 mg protein per ml and was not haemolytic against human erythrocytes.

DISCUSSION

This study shows that we were unable to obtain purified δ -haemolysin by employing some of the methods reportedly capable of yielding such preparations (Wiseman and Caird, 1968; Kreger *et al.*, 1971; Kantor *et al.*, 1972). In fact, with one procedure our final product did not contain demonstrable δ -haemolysin when tested by the electrophoretic localisation technique.

In comparing the three methods of purification, we followed as closely as possible the procedures described by the original investigators. The only exceptions were the choice of strain of *S. aureus* and the procedure used for preparation of the cell-free filtrates. These differences of procedure, however are not in themselves likely to account for our failure to obtain purified preparations of δ -haemolysin by the three procedures tested. Instead, we assume that our failure reflects our use of direct and reproducible criteria that involve the electrophoretic identification of δ -haemolysin (Haque, 1967) and the use of sensitive biological tests, as opposed to immunological and physico-chemical tests for the identification of the various fractions and the assessment of their purity.

Our choice of the 146P strain of *S. aureus* instead of the Wood 46M strain used by Kreger *et al.* (1971) and Kantor *et al.* (1972), and the strain E-delta used by Wiseman and Caird (1968), was based on studies that we previously conducted (Ali and Haque, 1974) wherein 13 selected strains of *S. aureus*

TABLE III

The biological and haemolytic activities demonstrable in fractions obtained from crude preparations of staphylococcal δ -haemolysin obtained by the method of Wiseman and Caird (1968)

Fraction	Size (mm) of zone of the stated activity														Haemolysins detected electrophoretically
	Lipase	Egg-yolk factor	Esterase	Staphylokinase	Lysozyme	Caseinase	Deoxyribonuclease	Penicillinase	Phosphatase	Hyaluronidase	Lysis of human RBC	Lysis of horse RBC	Lysis of rabbit RBC	Lysis of sheep RBC	
1. Crude filtrate	20	18	22	15	10	10	20	14	18	14	15	14	18	13	α δ
2. Dialysis of crude filtrate against 0.05M acetate buffer															
(a) supernate	15	8	5	15	10	10	18	14	—	4	6	6	8	6	δ (weak)
(b) precipitate	14	12	16	—	5	4	13	14	13	—	14	9	13	18	α δ
3. 2(b) dialysed against phosphate buffer, pH 7.0, and centrifuged:															
(a) supernate	14	8	10	—	4	4	14	13	12	—	12	4	4	4	α δ
(b) precipitate	—	—	—	—	—	—	5	—	—	—	7	7	8	5	δ
4. 3(a) applied to hydroxyl-apatite column															
(a) material not adsorbed	13	12	8	—	—	5	7	13	4	—	8	6	6	6	α δ
(b) material eluted with 2M NaCl	13	7	8	—	4	—	8	14	13	—	10	—	5	4	α δ
5. 4(b) applied to DEAE-column															
(a) material appearing in the void volume	—	—	—	—	—	—	4	14	5	—	—	—	5	—	α
(b) subsequent product eluted with															
(1) 0.1M phosphate buffer	—	4	—	—	4	—	4	—	—	—	—	—	4	—	—
(2) 0.5M phosphate buffer	10	5	5	—	—	—	—	—	5	—	—	—	—	—	—

— = No zone, or no haemolytic activity.

were compared for their ability to produce various haemolysins and other biologically active extracellular products. Of the 13 strains, the two best suited for the production and purification of δ -haemolysin were the 146P and the Wood 46M strains. Strain E-delta was considered unsatisfactory as it was found to produce α - and δ_1 -haemolysins.

Our choice of the method to produce cell-free filtrates was also based on studies that we previously conducted (Murphy and Haque, 1971). The dialysis-membrane procedure that we employed is capable of yielding concentrated preparations of crude δ -haemolysin and thus obviates the need for further concentration before the purification procedures.

This study indicates the need for developing new methods, or for refining the existing methods, for purifying δ -haemolysin, and it points out the complexity of the work in this field. There is a clear requirement for standardisation of

methods for (a) the identification of various products, and (b) establishing the purity of the final products. The simple determination of haemolytic titres and the interpretation of the zones of haemolysis on blood-agar plates cannot be relied upon to identify a given product to the extent that such methods have been used in the past. Additional methods such as the electrophoretic localisation technique (Haque, 1967), enzymophoresis (Abramson and Friedman, 1967), and techniques using disk electrophoresis and isoelectric focusing (Wadstrom, Thelestam and Mollby, 1974), should be employed to establish the identity of the test product.

Immunological and physicochemical methods, which have been used in the past to determine the purity of the final product, are of limited reliability as they are able to detect only the major components of a given preparation. For example, immunological methods such as the Ouchterlony immunodiffusion analysis will not detect antigenic impurities unless these are in a state of optimal proportion with their antibodies in the antiserum. Furthermore, the concentration of each antigen-antibody system has to be such as to produce a separate line for each system. This is unlikely to occur in a complex system such as is represented by the extracellular filtrates of staphylococci. Consequently, the Ouchterlony, and even the immuno-electrophoretic analyses, useful as these methods undoubtedly are, should not be solely relied upon to define the purity of a given product. At best, these methods should not be expected to detect impurities unless they are present at concentrations of 10–15 mg per ml.

Likewise, to be detected by analytical centrifugation, the concentration of the impurity has to be more than 1 mg per ml unless the impurity can be detected by a sensitive and accurate biological test (Schachman, 1966). The disk electrophoresis procedure is capable of detecting impurities in a concentration of as little as 1 μ g per ml (Cooksey, 1971), but even this procedure should not be the sole criterion of purity: crude unconcentrated culture filtrates of staphylococci seldom contain more than 5.5 mg of solids of microbial origin per ml or not more than 4 mg of protein per ml. The protein components of these solids represent at least 15 different biologically active products and a host of other products of unknown biological activity (Bernheimer and Schwartz, 1961). Accordingly, it is essential to establish alternate and more vigorous criteria for establishing the purity of staphylococcal products. The determination of biological activities known to be due to products other than the one being purified is a useful approach, as it may be possible to detect picogram quantities of certain biologically active material (Lachica, Genigeorgis and Hoeprich, 1971). Our study suggests that this approach merits more consideration by investigators concerned in the purification of staphylococcal products.

SUMMARY

Three methods at present available for the purification of staphylococcal δ -haemolysin were compared as to the purity and identity of the product obtained. None yielded a pure preparation of δ -haemolysin; one of the three

preparations did not contain demonstrable δ -haemolysin when tested electrophoretically, but it contained deoxyribonuclease, penicillinase, phosphatase and α -haemolysin. The second preparation had δ -haemolysin activity and was free of α -haemolysin, but it contained lipase, egg-yolk factor, esterase, deoxyribonuclease, penicillinase, phosphatase and hyaluronidase. The third preparation contained all of the products mentioned above, except phosphatase, and it also contained α -haemolysin, staphylokinase, lysozyme and caseinase. These findings are discussed with special reference to the requirement for criteria of purity in work with staphylococcal products.

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