PARTITIONING OF STAPHYLOCOCCAL δ HAEMOLYSIN

R. A. MURPHY AND R. HAQUE

Departments of Oral Diagnosis and Microbiology, University of Illinois
at the Medical Center, PO Box 6998, Chicago, Ill. 60680, USA

Staphylococcal δ haemolysin is considered important in staphylococcal pathogenicity because of its ability to lyse human red and white blood cells and its lethality in animal tests. Despite reports to the contrary (Wiseman and Caird, 1968; Kreger et al., 1971; Kantor, Temples and Shaw, 1972), it has neither been adequately purified nor characterised (Fackrell and Wiseman, 1974; Lee et al., 1976). Duplication of published purification procedures yielded preparations of δ haemolysin which, when analysed by sensitive biological and biochemical tests (Madler, Lee and Haque, 1976), were found to contain other biologically active products (Lee et al., 1976). Thus, characteristics ascribed to δ haemolysin have to be accepted with caution.

Crude preparations of δ haemolysin contain many biologically active substances. In purifying one component of such a mixture, it is a gross oversimplification of the problem to take as the major criterion of purity an increase in specific activity or the elimination of extraneous protein. The finding of many contaminating, biologically and biochemically active moieties in reportedly "pure" preparations emphasises the importance of knowing that these contaminating activities are eliminated by a chosen purification scheme. An understanding of the behaviour of these additional substances during various fractionation procedures is necessary for interpreting the effectiveness of any purification scheme.

Materials and methods

Crude δ haemolysin. The production, identification, and characterisation of the crude toxin and the analysis for biological activities of the preparations obtained by the various fractionation procedures were carried out as previously described (Haque, 1967; Murphy and Haque, 1967, 1971, 1974; Murphy, 1972; Lee et al., 1976; Madler et al., 1976).

Precipitation of δ haemolysin. Crude toxin was precipitated by taking portions of it and adding either 5 parts by volume of Universal Buffers (Britton and Welford, 1937) of pH 2.42, 2.9, 3.92, 4.4 and 4.82; a saturated solution of ammonium sulphate which yielded final saturations of 20%, 25%, 33%, 50%, 67% and 75%; or absolute ethanol to yield final concentrations of 5%, 10%, 15%, 20%, 25%, 33%, 50%, 67% and 75%. Before analysis, the supernates and the precipitates, after being dissolved in phosphate-buffered saline (PBS), were dialysed against PBS, lyophilised, and reconstituted with distilled water to the original volume of the crude toxin.

Molecular sieve chromatography. Sephadex G-100 (Pharmacia Inc., Piscataway, NJ, USA) was suspended in PBS and packed to a bed-height of 38 cm in a 2.5-cm x 45-cm glass column (Pharmacia) with a void volume of 55 ml. Sephadex G-200 was suspended in PBS and packed to a bed-height of 80 cm in a 2.5-cm x 90-cm glass column with a void volume of 42 ml. A 4-ml
sample of crude δ haemolysin was added to one G-100 column. An 8-ml sample of crude haemolysin was dialysed against PBS and 4 ml each were added to columns of Sephadex G-100 and G-200. All columns were developed at 4°C. G-100 columns had a flow rate of 27.6 ml/h and 4.6-ml fractions were collected. G-200 columns had a flow rate of 9.0 ml/h and 3.0-ml fractions were collected.

Ion-exchange chromatography. DEAE-Sephadex A-50 and CM-Sephadex C-50 (Pharmacia) were packed to a bed-height of 17 cm in 2.5-cm x 45-cm glass columns. Crude δ haemolysin was equilibrated with the starting buffers by dialysis and 4.0-ml portions were added to the columns.

Stepwise elutions were carried out under a constant pressure head. DEAE-Sephadex columns were developed at pH 7.0 with 0.02M phosphate buffer as the starting buffer. For elution, 0.005M, 0.01M, 0.015M, 0.02M, 0.03M, 0.08M, 0.28M, 0.48M and 0.98M sodium chloride were added to this starting buffer. CM-Sephadex columns were developed at pH 6.0 with a 0.05M phosphate starting buffer. Eluting buffers were 0.01M, 0.02M, 0.05M, 0.075M, 0.1M, 0.125M, 0.15M, 0.2M and 0.25M phosphate buffers, as well as 0.25M phosphate buffer containing 0.05M, 0.1M, 0.15M, 0.2M, 0.25M and 1.75M sodium chloride. Fractions of 4.0 ml were collected.

Hydroxylapatite chromatography. A suspension containing 2 g of hydroxylapatite (Bio-Rad Laboratories, Richmond, Calif., USA), previously equilibrated with the starting phosphate buffer (0.05M), was added to a 45-mm-diameter stainless steel pressure-filter holder (Gelman Instrument Co., Ann Arbor, Mich., USA) fitted with a 0.8 μm membrane filter (Millipore Corp., Bedford, Mass., USA). The top of the filter holder was connected to a regulated pressure source and the buffer removed by applying pressure to the column. A 4.0-ml quantity of crude δ haemolysin, previously equilibrated with the starting buffer, was mixed with the hydroxylapatite and allowed to equilibrate for 15 min. Pressure was then applied, and the fluid containing the unabsorbed material was forced from the bed. The entire quantity of the desired eluting buffer was then added above the bed and sufficient pressure applied to obtain a flow rate of 1 ml/min. Fractions of 4.0 ml were collected. The buffers used were 0.005M, 0.06M, 0.12M, 0.25M, 0.4M, 0.5M, 0.6M, 0.7M, 0.8M, 0.9M, 1.0M and 4.0M sodium-sodium phosphate. The 0.005M and 0.06M buffers also contained 0.15M sodium chloride.

RESULTS

Biological and biochemical activity of crude δ haemolysin

The crude toxin was golden yellow and had an intense pungent odour. It lysed human, horse, rabbit, and sheep erythrocytes with titres of 256, 128, 2048, and 64 respectively, and contained hyaluronidase, lipase, esterase, egg-yolk factor, lysozyme, nuclease, gelatinase, caseinase, and usually staphylokinase. Detectable quantities of coagulase, penicillinase and phosphatase were not present.

Precipitation

Toxin-buffer mixtures with a pH of 3.36 or lower yielded precipitates when centrifuged at 2000 g. At pH 3.36, lysozyme remained completely in the supernate. Extracts of the precipitate, made with PBS (pH 7.2) and with buffers of pH 7.3 and 10.5, all contained α and δ haemolysins, lipase, esterase, egg-yolk factor and traces of nuclease. When the precipitate remaining after extraction at pH 7.3 was further extracted with buffer pH 10.5, it dissolved completely and yielded additional quantities of these same activities. Gela-
tinase and caseinase were not found in the supernate after precipitation at pH 3.36 or in any of the extracts.

After the addition of ammonium sulphate, precipitation began to occur in the crude toxin at 25% saturation and was nearly maximal at 50% saturation. The δ haemolysin appeared in the precipitate at 25% saturation and was totally precipitated at 50% saturation. The precipitate obtained at 50% saturation contained all the δ haemolysin, esterase, and egg-yolk factor, portions of the α haemolysin, lipase, lysozyme, gelatinase, and caseinase, but none of the nuclease, which did not precipitate until 67% saturation.

Ethanol concentrations of 33% or higher yielded a heavy precipitate, with the δ haemolysin remaining completely in the supernate at all concentrations. The supernates from samples showing maximal precipitation contained δ haemolysin, lipase, esterase, egg-yolk factor, gelatinase, nuclease, and staphylokinase, while the precipitates contained α haemolysin, lipase, esterase, lysozyme, gelatinase, and caseinase.

**Chromatography**

*Sephadex G-100 chromatography* (fig. 1) yielded seven peaks of proteinaceous material. δ Haemolysin was contained in the first peak, which also contained lipase, esterase, egg-yolk factor and hyaluronidase. This was followed by three small peaks. The first of these lysed rabbit erythrocytes and contained lipase, esterase, egg-yolk factor, hyaluronidase and lysozyme. The second lysed human and rabbit erythrocytes and contained lipase, esterase, hyaluronidase, lysozyme and nuclease. The third small peak lysed rabbit erythrocytes and contained hyaluronidase, nuclease, and staphylokinase. These three peaks contained δ haemolysin but no δ haemolysin. Three fractions which appeared immediately after these peaks contained the caseinase. These were followed by three large peaks of proteinaceous materials. The first and second contained the odoriferous material and also caused gradual lysis of human and rabbit erythrocytes. This haemolytic ability, as well as the odour, was lost when the samples were lyophilised. The third peak did not show any of the activities tested for. These three large peaks and the odour were absent if the toxin was exhaustively dialysed. The peak containing the δ haemolysin, which appeared immediately after the void volume, had a specific activity (haemolytic units/mg of protein) of 45. It represented a 91% recovery of δ haemolysin with a 3-fold purification.

*DEAE-Sephadex chromatography* (fig. 2) resulted in the adsorption of the δ haemolysin and a portion of lipase. Both of these were eluted in four peaks
(I-IV) with 0.04M, 0.05M, 0.1M and 0.3M buffers. The α haemolysin, egg-yolk factor, hyaluronidase, lysozyme, and the remainder of the lipase failed to be adsorbed. The fractions forming each of the four peaks were separately pooled, lyophilised, and rehydrated to a final volume of 4.0 ml each. The most purified δ haemolysin was contained in peak I, which represented a recovery of 6.2% with a 1.9-fold purification. Peaks II, III, and IV also contained δ haemolysin. Collectively these four peaks represented a recovery of 37.5% of the δ haemolysin with a 1.1-fold purification.

**CM-Sephadex chromatography** (fig. 4) yielded at least seven peaks of proteinaceous material with δ haemolysin in five of these. A small amount of δ haemolysin remained unadsorbed while the majority was adsorbed, and was eluted with the 0.1M, 0.2M 0.25M and 0.3M buffers. The α haemolysin and hyaluronidase were eluted entirely with the 0.1M and 0.125M buffers. A small portion of the lipase remained unadsorbed while the remainder was eluted in
STAPHYLOCOCCAL δ HAEMOLYSIN

FIG. 2—Molecular sieve chromatography of dialysed crude δ haemolysin on Sephadex G-200. See legend for fig. 1.

FIG. 3.—Chromatography of crude δ haemolysin on DEAE-Sephadex at pH 7.0. See legend for fig. 1.
Fig. 4.—Chromatography of crude δ haemolysin on CM-Sephadex at pH 7.0. See legend for fig. 1.

Fig. 5.—Chromatography of crude δ haemolysin on hydroxylapatite at pH 6.8. See legend for fig. 1; Elect = results of electrophoretic localisation.
buffers in the range 0.2M–0.45M. The lysozyme was eluted at 0.3M and 0.35M. The fractions from each of the five haemolytic peaks were individually pooled, lyophilised, and rehydrated to a final volume of 4.0 ml. Material eluting at 0.1M contained 50% of the $\delta$ haemolysin and represented a purification of 13-fold. While it did contain $\alpha$ haemolysin and hyaluronidase, it was free from lipase. Peaks 3, 4, and 5 did not contain any $\alpha$ haemolysin but contained lipase. Collectively, peaks 2, 3, 4 and 5 represented a recovery of 80% with a purification of c. 8-fold.

*Chromatography of crude dialysed toxin on hydroxylapatite* (fig. 5) yielded numerous peaks of proteinaceous material. Eight peaks of human-cell-lysing activity were eluted in the range 0.25– and 1.0M. Rabbit-cell-lysing activity appeared in these same eight peaks and was eluted with the 0.06M and 0.12M buffers. Alpha haemolysin was eluted with the 0.06M and 0.12M buffers, $\alpha$ and $\delta$ haemolysins in the 0.25M and 0.4M buffers, and only $\delta$ haemolysin in the 0.5M and succeeding buffers. Lipase was eluted in all buffers from 0.25M to 1.0M, hyaluronidase between 0.06M and 0.25M, and lysozyme with the 0.12M buffer. Collectively, all peaks containing $\delta$ haemolysin showed a complete recovery of this toxin with a 4-fold purification.

**DISCUSSION**

The data presented here indicate the inability of conventional methods of fractionation to separate $\delta$ haemolysin completely from other components of crude culture filtrates. In addition, the chromatographic procedures employed frequently yielded multiple peaks of $\delta$ haemolysin, while crude preparations yielded only one peak of $\delta$ haemolysin when tested electrophoretically and by molecular sieving. These findings, as well as those previously reported (Fackrell and Wiseman, 1974; Lee et al., 1976) clearly indicate the need to renew efforts to develop methods for the purification of $\delta$ haemolysin. During the development of such methods, due attention must be given to the criteria for identifying the $\delta$ haemolysin, as well as to methods for detecting the presence of impurities in the isolated fractions. We feel that electrophoretic methods (Kayser and Raynaud, 1965; Lochmann et al., 1969; Murphy and Haque, 1971; Wådstrom, Thelestam and Möllby, 1974) should be used for the former purpose and biological methods (Madler et al., 1976) for the latter.

**SUMMARY**

The behaviour of the biologically active components present in crude $\delta$ haemolysin was followed during various fractionation procedures utilised in the purification of $\delta$ haemolysin. Most chromatographic techniques yielded multiple peaks of $\delta$ haemolysin. None of the procedures completely separated $\delta$ haemolysin from the other components of crude culture filtrates. Efforts to purify $\delta$ haemolysin should be renewed.

This investigation was supported by USPHS Grant no. AI-08906 from the National Institute
of Allergy and Infectious Diseases and USPHS General Research Support Grant no. 5501-RR05309-11.

REFERENCES


