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A Physico-chemical Study on the Haemolytic Activities of *Staphylococcus aureus*

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In order to obtain good yields of haemolysins from *Staphylococcus aureus* (238) the cells have to be cultivated for rather long times. In the process heavily charged acid polyelectrolytes appear in the medium, most probably due to lysis of part of the cells. Whether the haemolytic activities are to be purified from the cells or, as in the following report, from the medium, these acid polyelectrolytes will interfere with the purification procedure^{1,2}. Some results of a characterization by means of counter flow electrophoresis² and chromatography will be reported.

Materials and methods. *Medium I.* 1) Heat 1 800 g of freshly ground horse meat in 3 000 ml water to 75°C. 2) Add 3 000 ml 0.1 M sodium carbonate. 3) Allow to cool to 45–50°C. 4) 5 g pancreatine and 30 ml CHCl_3 are added. 5) Let stand for 3.5 h at 37°–40°C. 6) Add 40 ml conc. HCl. 7) Heat to boiling. 8) Filter through coarse filter paper (not cotton wool). 9) Adjust

pH to 8.2–8.3 with 0.1 M HCl and 0.1 M NaOH. 10) Heat to boiling and filter again. 11) Add agar to 1.2 % W/v. 12) Autoclave for 30 min at 119°C. 13) Filter. 14) Autoclave for 20 min at 119°C. 15) Dissolve one part agar medium (I) in 1.34 parts broth medium (II, see below). This step gives a final agar concentration in medium I of 0.85 %. 16) Dispense and autoclave for 15 min at 119°C.

Medium II. 1) Autoclave together 2 500 g of freshly ground horse meat and 6 000 ml 0.25 M NaCl for one hour at atmospheric pressure and filter through cheese cloth and coarse filter paper. 2) Make up volume to 6 000 ml. 3) Add peptone (Merck) to give 4 % W/v. 4) Adjust pH to 7.4 (see step 9 for medium I). 5) Heat to boiling and filter through coarse filter paper. 6) Add 12 g KH_2PO_4 per litre. 7) Adjust pH to 7.4. 8) Boil the broth for 30 min, filter and autoclave for 20 min at 119°C. 9) Dispense and autoclave for 45 min at 100°C.

Cultivation technique. The cells were first cultivated on 10 % ox blood agar plates for 24 h at 37°C. No carbon dioxide was added to the air. The cells were transferred to a 10 % horse serum broth and cultivated for 24 h under the same atmospheric conditions.

From this culture 5 ml were inoculated into 400 ml of the semisolid medium I, contained in a 2 litre Fernbach bottle. After 72 h, 600 ml of medium II were added to the bottle, and the cultivation was continued for another 72 h. The temperature was 37°C and the gas phase consisted of $\text{CO}_2:\text{O}_2$ in the proportions 2:5. The gas phase was regulated by two narcose flowmeters (AGA 67827K and AGA 67828K, respectively) coupled in parallel.

Harvesting procedure. The culture was cooled rapidly to +1°C and was centrifuged at +1°C for 90 min at $7\,000 \times g$ average.

Concentration of the medium. Different methods were tried. Freeze drying, pervaporation, the polyethylene glycol method of Kohn³ and the technique of Palmstierna⁴ were used. In our hands the last mentioned method gave the best results. Very small, if any, losses in haemolytic (*in vitro*⁵) and lethal activity (mice) were found. The lethal activity was tested on 12–14 g mice (Sigtuna strain) by giving 0.25 ml intraperitoneally. Serial dilutions were made in saline.

Chemical methods. In order to isolate the proteins from the acid polyelectrolytes under as mild conditions as possible, the counter flow electrophoresis (CFE) method of Eliasson *et al.*² was used. In this case the proteins were sucked off from the top of the column and pumped into a jacketed and cooled DEAE ion exchanger column⁶ (2 × 20 cm) *via* a modified hose pressure micropump². The electrophoresis

Fig. 1. Counter flow electrophoresis in combination with a DEAE column (2×20 cm, see text). The electrophoresis was finished at tube No. 65. Thereafter the DEAE was eluted with increasing concentration of NaCl in buffer. *Haemolytic activity* (tubenumbers: 25–28; black).

Lethal activity (tubenumbers: 25–31; striped).

was performed in 0.01 M Na-phosphate buffer (pH 6.85). The DEAE was in the phosphate form and equilibrated with the buffer mentioned.

The active fractions (*cf.* Fig. 1) were cut out and loaded on CMC-cellulose⁶ or on cellulose esterified with succinic anhydride⁷. The pK in water of these exchangers were 3.8 and 7.6, respectively.

Concentrated medium was loaded on Sephadex G 25, G 50 and G 75⁸ jacketed and cooled columns, equilibrated with water. The columns were eluted at a rate of 1 ml per cm² and hour.

Paper chromatography for amino acids was performed on 18 h hydrolysates in 6 N HCl. In the first dimension butanol:glacial acetic acid:H₂O, 4:1:5 v/v, upper phase, was used. In the second dimension pyridine:H₂O, 8:2 v/v, was used.

Results and discussion. The haemolytic (*in vitro*) as well as the mouse lethal activity passed out from the top of the counter flow electrophoresis column in the front of the proteins. The toxins thus migrate like positively charged proteins.

The toxins are not retarded by DEAE at the pH and the ionic strength used (spec.

conductivity: $1.18 \times 10^{-3} \Omega^{-1} \text{ cm}^{-1}$). Since DEAE is an anion exchanger, the toxins are positively charged (or lacking ionic groups).

The toxic activities, purified by electrophoresis and passage through DEAE, were firmly fixed on cation cellulose exchangers. Succinate esterified cellulose (SEC) and CMC firmly fixed the toxins. They could not be eluted even with phosphate buffered 2 M NaCl, pH 6.85. The toxins were thus either destroyed or irreversibly fixed on these celluloses. Such a fixation indicates a high pK value of the toxins.

It is known that Sephadex retards basic materials under the conditions used⁹. Active material was retarded on Sephadex.

The toxins do not pass dialysis membranes. They migrate rapidly towards the cathode. They are not retarded on the DEAE-cellulose but they are retarded on Sephadex. They are almost irreversibly bound to CMC and SEC. Paper chromatography indicates that basic amino acids are represented in rather high proportion. The toxins may thus be associated to high molecular weight substances with dominating basic properties.

Work is continuing towards a better definition of these substances.

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