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The hydrophobic character of thiol-activated cytolysins

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Hydrophobic chromatography on phenyl–Sepharose has revealed the decidedly hydrophobic character of several members of the group of cytolytic proteins termed 'thiol-activated'. Pneumolysin, alveolysin, cereolysin, and streptolysin O were found to be equally hydrophobic, as were the oxidized and reduced forms of alveolysin. Hydrophobic chromatography has been utilized in the development of an improved procedure for the purification of pneumolysin.

The thiol-activated ('sulphydryl-dependent') cytolysins comprise a group of related proteins produced by Gram-positive bacteria. All these agents are active only in the reduced state, show cross-neutralization and are inhibited by small quantities of cholesterol (Smyth & Duncan, 1978). They lyse only cells with cholesterol-containing membranes, and cholesterol appears to be the lysin receptor (Johnson *et al.*, 1980). Their ability to interact with membranes suggests that these proteins might be expected to have some hydrophobic character. We have studied four members of the group (alveolysin, cereolysin, streptolysin O and pneumolysin) in order to evaluate and compare their hydrophobicity and to determine whether oxidation of the lysin results in a modification of the hydrophobic character.

Experimental

Streptococcus pneumoniae (R36A, from the A.T.C.C.) was grown in peptone medium and harvested as described previously (Johnson, 1977). Cell suspensions were stored at −40°C. Cells autolysed upon thawing, and disruption was completed by three 5 s sonications (Sonifier Cell Disrupter), followed by centrifugation at 12000 ***g*** for 1h. The crude extract was 58% saturated with $(NH_4)_2SO_4$ in an ice bath and stirred for 30 min. The precipitate was collected by centrifugation, dissolved in the smallest possible volume of 0.02 M-NaCl in 0.01 M-sodium phosphate buffer, pH 7.0 (starting buffer) and dialysed for 2h against the same buffer. For preparation of the other thiol-activated lysins, the same medium was employed, utilizing a 2% (v/v) inoculum of an overnight culture of *Bacillus cereus* (stock collection), *Bacillus alvei* (A.T.C.C. 6344), or *Streptococcus pyogenes* (B703, Streptolysin S$^-$, kindly supplied by Dr. Joseph Alouf, Institut Pasteur, Paris, France). *B. alvei* and *S. pyogenes* were grown in 100 ml quantities contained in 125 ml flasks without agitation. *B. cereus* was grown in 100 ml quantities in 1 litre flasks on a rotary shaker. After incubation at 37°C for 16h, cells were removed by centrifugation and $(NH_4)_2SO_4$ added to 60% saturation (80% for *S. pyogenes*). After being left overnight at 4°C, the precipitates were collected by centrifugation, dissolved and dialysed as described above. Protein was determined by the method of Lowry *et al.* (1951).

The haemolysin levels were measured and calculated in haemolytic units as described previously (Johnson, 1972), with the exception that buffers used for assay of cereolysin contained 5% (v/v) glycerol in place of 0.1% bovine serum albumin. The haemolysin content of column fractions was determined more simply by adding 10–20 μl of the fraction to 0.5 ml of assay buffer, followed by the addition of 0.5 ml of a 1.25% (v/v) erythrocyte suspension. Relative haemolytic activity was then estimated from the time required for complete lysis to occur. When necessary, the preparations were reduced before titre assessment by addition of β-mercaptoethanol to a final concentration of 0.03 M, followed by incubation at room temperature for 15 min.

Hydrophobic chromatography was performed at 4°C with phenyl–Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.), with either a 10 ml (0.9 cm × 16 cm) or a 200 ml (2.5 cm × 40 cm) column prepared in 0.02 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.0. Batch elutions were made with: 2 column vol. of the starting buffer; 1 vol. of 10 mM-NaCl in 10 mM-buffer; 1 vol. of 5 mM-NaCl in 1 mM-buffer; and 2 vol. of deionized water (pH 7.0, conductance 9.1 μS). Plastic tubes were used for collection of fractions and for

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preparation of dilutions for titre assessment. For chromatography with hydroxyapatite, 11g of Bio-Gel HTP (Bio-Rad Laboratories, Richmond, CA, U.S.A.) was prepared in 1mM-sodium phosphate buffer, pH6.8, and used in a 1.7-cm diameter column. Elution was effected with a gradient of 150ml each of 10mM- and 0.24M-buffer, pH6.8. Polyacrylamide-gel electrophoresis was performed as described previously (Johnson, 1972).

Results

Chromatography of the $(NH_4)_2SO_4$-precipitated fraction of *B. alvei* culture supernatant fluid on phenyl–Sepharose revealed (Fig. 1) that very little of the protein was adsorbed under the conditions utilized. Alveolysin, however, was strongly adsorbed and was eluted (with a recovery of about 40%) only when deionized water was employed as eluent. The fractions were analysed before and after reduction to determine whether lysin in the oxidized state (the activity of which could be observed only after reduction) would be eluted under the same conditions as the spontaneously reduced alveolysin. Fig. 1 shows that that was indeed the case, as only a single peak was observed before and after reduction.

Cereolysin [precipitated with $(NH_4)_2SO_4$ from culture supernatant fluids of *B. cereus*] was also strongly bound by phenyl–Sepharose. This lysin was eluted under the same conditions (results not shown) as alveolysin, with a somewhat lower recovery (about 30%). In the case of Streptolysin O, a similar elution pattern was obtained, but recovery was very low (2–3%). Efforts to improve the recovery by addition of 0.01M-EDTA to sample and eluents in an attempt to inhibit possible proteinase activity were unsuccessful; in fact, no loss of lytic activity in the $(NH_4)_2SO_4$-precipitated fraction was observed during incubation at room temperature for 3h, even when 0.03M-β-mercaptoethanol was added. Attempts to elute more lysin with 50% (v/v) ethylene glycol were unsuccessful.

Results obtained on chromatography of pneumolysin with phenyl–Sepharose are shown in Fig. 2. In this case the lysin is cell-associated; thus fractionation with $(NH_4)_2SO_4$ was performed on a crude cell extract rather than a culture supernatant fluid, and more protein was applied than was used in the experiments described above. Again, by far the greater part of the protein applied to the column was not adsorbed. Pneumolysin was, in common with the other lysins tested, strongly adsorbed and was eluted only after the application of deionized water. In an attempt to utilize hydrophobic chromatography as a method for purification of pneumolysin, the procedure was scaled up 20-fold. Since, as can be seen in Fig. 2, the peak of protein-containing material was

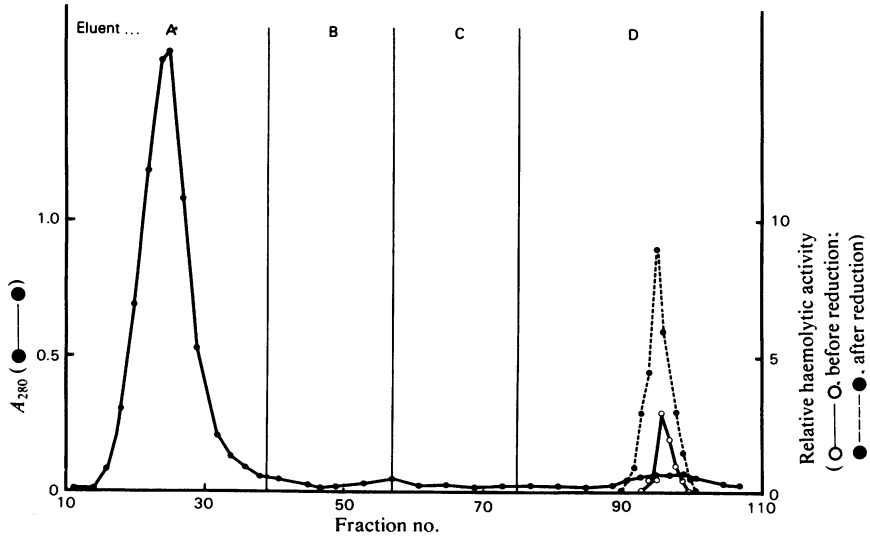

Fig. 1. *Chromatography on phenyl–Sepharose of $(NH_4)_2SO_4$-precipitated culture supernatant fluid of Bacillus alvei*
Eluents: A, 20ml of 0.02M-NaCl in 0.01M-sodium phosphate buffer, pH7.0; B, 10ml of 0.01M-NaCl in 0.01M-buffer; C, 10ml of 5mM-NaCl in 1mM-buffer; D, 20ml of deionized water, pH7.0. A sample (1ml) containing 20000 haemolytic units was applied to the column.

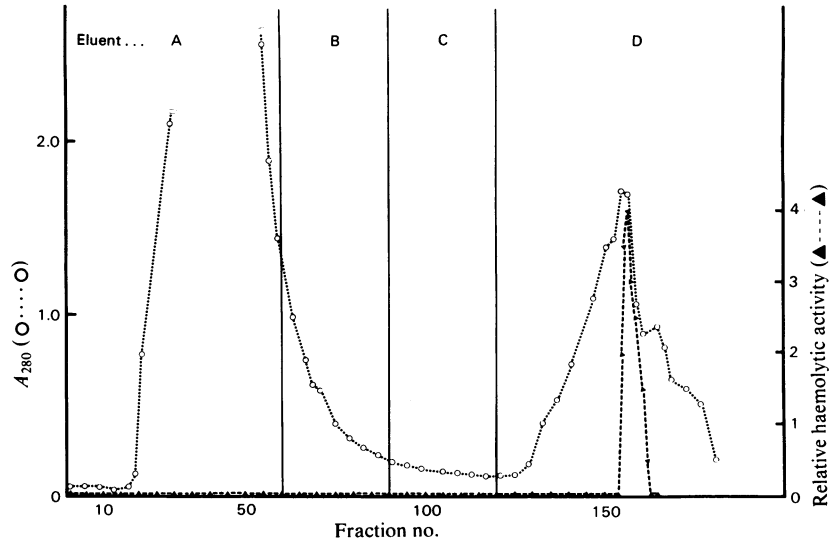

Fig. 2. *Chromatography on phenyl–Sepharose of $(NH_4)_2SO_4$-precipitated extract of S. pneumoniae*
Eluents: A, 20ml of 0.02M-NaCl in 0.01M-sodium phosphate buffer, pH7.0; B, 10ml of 0.01M-NaCl in 0.01M-buffer; C, 10ml of 5mM-NaCl in 1mM-buffer; D, deionized water, pH7.0. A sample (1ml) of material containing 150000 haemolytic units was applied to the column.

Table 1. *Purification of pneumolysin*

Fraction	Volume (ml)	Protein (mg)	Haemolytic units	Specific activity*	Purification (fold)
Crude extract†	64	1101	1.0×10^7	9.1×10^3	1
$(NH_4)_2SO_4$ ppt.	23	527	8.5×10^6	1.6×10^4	1.8
Phenyl–Sepharose eluate	27	62	7.9×10^6	1.3×10^5	14
Hydroxyapatite eluate	4	5.3	5.1×10^6	9.6×10^5	105

* Haemolytic units/mg of protein.
† Prepared from cells harvested from 8 litres of culture.

much broader than that of eluted lysin, this step was followed by chromatography on hydroxyapatite. The results of this procedure are presented in Table 1. It can be seen that a good recovery (93%) of the lysin applied to the phenyl–Sepharose column was obtained. The final product (after chromatography on hydroxyapatite) represented a yield of 51% of the lysin present in the crude extract. Examination of this material by polyacrylamide-gel electrophoresis showed a single major protein band and two faint minor bands.

Discussion

The present results demonstrate that the cytolytic agents alveolysin, cereolysin, streptolysin O and pneumolysin have substantial hydrophobic character, since they were, unlike most of the proteins in the samples, bound to phenyl–Sepharose at a relatively low ionic strength (0.02M-NaCl in 0.01M-sodium phosphate) and eluted only when deionized water was used as eluent. All of the lysins were eluted at the same point and are thus judged to be of equal hydrophobicity. Since oxidized and reduced alveolysin were eluted at the same point, it seems likely that the hydrophobic character of the molecule is not modified by the reversible oxidative reaction which does, however, prevent fixation of lysin to target cells (Smyth & Duncan, 1978).

Recovery of pneumolysin from the phenyl–Sepharose was sufficiently good that it was successfully utilized for purification of this protein (Table 1), in a procedure that is simpler and more efficient than that we have used prevously (Johnson *et al.*, 1980).

The reason for the poor recovery of streptolysin O was not clear, although lysin destruction by proteinase action was shown to be unlikely. It is possible that there was heterogeneity in the streptolysin O molecules [as has been previously reported (Smyth & Duncan, 1978)] and that a major fraction was not eluted under the conditions used.

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