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Hydrophobic interaction chromatography for the purification of cytolytic bacterial toxins

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Abstract

The usefulness of hydrophobic interaction chromatography for the simple purification of cytolytic bacterial toxins was studied. Conditions are described for different hydrophobic interaction chromatographic media for purifying with high yields two different kinds of such haemolysins, the thiol-activated toxin listeriolysin O from *Listeria monocytogenes* and alpha-toxin from *Staphylococcus aureus*. For listeriolysin O, purification on butyl-Sepharose was followed by gel filtration chromatography. From butyl-Sepharose the recovery was 84%. The final product had a specific activity of 620 000 U/mg with an overall recovery of 22%. Alpha-toxin was obtained by a single purification step from alkyl-Superose with 80% recovery and a specific activity of 29 000 U/mg. On sodium dodecyl sulphate polyacrylamide gel electrophoresis purified listeriolysin O and alpha-toxin showed a single band. Another thiol-activated toxin, streptolysin O from group A streptococci, showed a recovery of 38% from butyl-Sepharose. The results suggest the feasibility of using hydrophobic interaction chromatography, particularly with columns of weak hydrophobicity, for the purification of bacterial haemolysins in high yield.

1. Introduction

Several pathogenic bacteria produce extracellular haemolysins as important virulence factors. Listeriolysin O, secreted by the intracellular bacterium *Listeria monocytogenes*, is a well characterized haemolysin contributing to its intracellular lifestyle [1]. After adherence of the bacterium to target cells and its phagocytosis, listeriolysin O promotes bacterial evasion from the endosome into the cytosol of the host cell [2–5]. Listeriolysin O is a member of the family of sulphydryl (SH)-activated bacterial toxins that are produced by various Gram-positive species with streptolysin O from group A streptococci as prototype. These toxins have a similar molecular mass of *ca.* 60 000 and their haemolytic activity is destroyed by exposure to oxygen and restored by addition of reducing agents such as cysteine. These toxins bind to cholesterol-containing membranes where they oligomerize to form arc- and ring-shaped multimeric structures containing up to 100 toxin monomers. They span the membrane and promote transition of large molecules through the membrane [6]. The alpha-toxin from *Staphylococcus aureus* represents a different type of haemolysin. This toxin is independent of sulphydryl and has a molecular mass of 34 000. It forms hexamers, causing small transmembrane pores in the target membrane. These pores of defined size allow the translocation only

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of small molecules such as amino acids, whereas larger entities such as proteins are retained by the membrane [7,8].

Haemolysins are also of great interest for numerous cell-biological studies. Because listeriolysin O is most active under weakly acidic conditions of about pH 6, its function can be modulated by pH changes [9]. This toxin is useful for studies concerned with protein translocation from acidified endosomal compartments to the cytosol, *e.g.*, studies of antigen presentation. The alpha-toxin is used for permeabilizing cell preparations, allowing studies on the influence of small molecules on cellular processes [10]. Because of the wide application of haemolysins in functional studies, their rapid purification is of increasing interest. So far sulphydryl-activated haemolysins have been purified by covalent chromatography on thiol-activated columns as a major purification step [11]. For the purification of alpha-toxin, the use of cation-exchange chromatography [12] and porous glass beads [13] has been described.

We were interested in purified listeriolysin O, for which covalent chromatography gives a low recovery [9]. In an attempt to obtain a high recovery of listeriolysin O and other cytolysins, we were looking for a simple alternative purification method: as haemolysins interact with hydrophobic membranes it appeared reasonable to exploit this feature for their purification by hydrophobic interaction chromatography (HIC). HIC columns bind proteins via hydrophobic patches on their surface. Parameters that influence this chromatographic process are HIC columns with ligands of different hydrophobic strength on the one hand, and variation of the hydrophobic character of the protein on the other. The method most widely used for increasing the hydrophobicity of a protein is the addition of antichaotropic agents such as ammonium sulphate [14]. Screening the literature for procedures based on HIC to purify cytolytical bacterial toxins, we found reports for the thiol-activated toxins pneumolysin from *Streptococcus pneumoniae* [15] and tetanolysin from *Clostridium tetani* [16], for extracellular cytolysins from halophilic bacteria [17,18] and for a cytolytic enterotoxin from *Bacteroides fragilis* [19]. Employing HIC for listeriolysin and alpha-toxin, we were successful in purifying haemolysins of scientific and technological interest in high yield by a one- or two-step procedure. Hence HIC may prove a simple and powerful method for purifying membrane-attacking bacterial toxins in general.

2. Experimental

2.1. Production of haemolysins

Listeriolysin O

From an overnight preculture of *Listeria monocytogenes* WTD [20] a 1% inoculum was grown overnight in 350 ml of medium (Bacto tryptic soy broth from Difco Labs., Detroit, MI, USA) in 1-l erlenmeyer flasks on a rotary shaker (120 rpm at 37°C). Bacteria were removed by centrifugation (20 min at 10 000 rpm, 17 000 *g*, and 10°C) and the supernatants were filtered through 0.2-μm membrane filters. This sterile supernatant was either used directly for chromatography or alternatively precipitated at 4°C with ammonium sulphate (80% saturation), and the collected precipitate was dissolved in 20 m*M* sodium phosphate (pH 6.5). Crude haemolysin stocks were stored frozen at −20°C until used.

Alpha-toxin

This toxin from *Staphylococcus aureus* (strain Wood 46, ATCC 10832, DSM 20491) was prepared according to Lind *et al.* [12]. Briefly, a 1% inoculum of an exponentially growing preculture was cultured overnight in 350 ml of medium (Bacto tryptic soy broth) in a 1-l indended erlenmeyer flask on a rotary shaker (150 rpm at 37°C). After 18 h, the bacteria were removed as described above and ammonium sulphate was added at 4°C to the supernatant (75% saturation). The haemolysin was solubilized from the precipitate with 10 m*M* sodium acetate–20 m*M* sodium chloride (pH 5.0) and stored frozen at −20°C in aliquots.

Streptolysin O

Streptolysin O was purchased from Diagnostics Pasteur (Marnes-la-Coquette, France). The material of one 25-ml vial was dissolved in 2.5 ml of 10 m*M* sodium phosphate (pH 7.0).

2.2. Chromatography

Alkyl-Superose HR5/5, butyl-Sepharose, phenyl-Sepharose Cl-4B, octyl-Sepharose Cl-4B, the Pharmacia HIC test kit, Mono S HR5/5, Mono Q HR5/5 and Superose 12 HR10/30 were obtained from Pharmacia Biosystems (Freiburg, Germany) and Econo-Pac methyl-HIC cartridges from Bio-Rad Labs. (Munich, Germany).

Listeriolysin O

Chromatography was carried out on a fast protein liquid chromatographic (FPLC) system (Pharmacia Biosystems) at room temperature. For HIC on butyl-Sepharose (5-ml column, 1-cm diameter) the material was adjusted to 27% saturation of ammonium sulphate. Either the culture supernatant was directly adjusted by adding ammonium sulphate or, alternatively, the fraction obtained after 80% ammonium sulphate precipitation was dissolved and dialysed in 20 m*M* sodium phosphate (pH 6.5) and then ammonium sulphate was added to 27% saturation. After loading on to the column equilibrated with 25% ammonium sulphate in 20 m*M* sodium phosphate (pH 6.5), washing was carried out with 25% ammonium sulphate in the same buffer because with 27% ammonium sulphate proteins occasionally precipitated on the column. Elution was performed with a decreasing linear gradient of ammonium sulphate from 25% to 0% in the same buffer. The flow-rate was 1 ml/min. Gel filtration was carried out with Superose 12 HR10/30 in phosphate-buffered saline (PBS) (50 m*M* sodium phosphate buffer containing 150 m*M* sodium chloride) of pH 6.0.

Alpha-toxin

All chromatographic steps were carried out at 4°C because at room temperature the recoveries dropped by about 40%. The toxin fraction after 75% ammonium sulphate precipitation in 20 m*M* sodium phosphate buffer (pH 7.0) was adjusted to 45% of ammonium sulphate and loaded on to an alkyl-Superose HR5/5 (1 ml) equilibrated with 45% ammonium sulphate in the same buffer. Elution was done with a decreasing linear gradient of ammonium sulphate in 20 m*M* sodium phosphate (pH 7.0). The flow-rate was 0.5 ml/min.

Streptolysin O

The conditions were identical with those described for alpha-toxin purification except that 35% instead of 45% ammonium sulphate was used as the initial salt concentration.

2.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Electrophoresis was carried out under reducing conditions in a discontinuous 12.5% polyacrylamide gel according to standard procedures in a mini-PROTEAN II electrophoresis system (Bio-Rad Labs.) and the gels were silver stained [21]. For immunological detection of specific bands, proteins were transferred to reinforced nitrocellulose (Schleicher and Schüll, Dassel, Germany) in a semi-dry blotting system. After blocking the membrane with 3% skim-milk powder (Fluka, Neu-Ulm, Germany) in 50 m*M* Tris–hydrochloric acid (pH 7.5), the mAb B8B20-3-2 [22] against listeriolysin O was used as first antibody (1 μg/ml). Further incubation steps were done with alkaline phosphatase conjugated goat anti-mouse IgG (Jackson Immuno Research Labs., West Grove, PA, USA). The blots were then developed with 0.8 μg/ml 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and 1.6 μg/ml 4-nitrotetrazolium blue chloride (both from Fluka) in 1 *M* diethanolamine–hydrochloric acid, 0.5 m*M* magnesium chloride (pH 9.8).

2.4. Determination of haemolysins

Listeriolysin O

The sample was serially diluted in PBS (pH 6.0) containing 0.1% bovine serum albumin and then activated by adding cysteine (20 m*M* final

concentration). Diluted samples (100 μl) were incubated at 37°C for 45 min with 50 μl of sheep erythrocytes ($6 \cdot 10^8$ cells/ml in PBS, pH 6.0 [23]) in U-bottomed 96-well plates. After centrifugation (1200 rpm, 5 min, 300 *g*) 50 μl of the supernatant were transferred into a flat-bottomed 96-well plate containing 50 μl of PBS per well. The concentration of haemolysin was determined spectrophotometrically at 405 nm in an enzyme-linked immunosorbent assay (ELISA) reader. For total haemolysis a concentrated sample of haemolysin was used. The dilution of haemolysin lysing 50% of erythrocytes was determined and the reciprocal value was used to calculate the haemolytic units (*U*) per millilitre of the undiluted sample.

Alpha-toxin

A 10-μl volume of serially diluted sample in PBS (pH 7.0) was incubated with 100 μl of rabbit erythrocytes ($2.5 \cdot 10^8$ cells/ml) at 37°C for 45 min in U-bottomed 96-well plates [12]. The subsequent procedure was as described above for listeriolysin O.

Streptolysin O

The assay was carried out as described for listeriolysin O but at pH 7.5 instead of pH 6.0.

2.5. Protein assay

The BCA test from Pierce (Oud Beijerland, Netherlands) was used with bicinchoninic acid as the test reagent [24]. Contaminating substances were removed by precipitating the proteins with trichloroacetic acid. The sample was mixed with at least 10% (v/v) of 3 *M* trichloroacetic acid, incubated for 5 min, centrifuged (15 min, 12 000 *g*) and the sediment was washed once with 200 μl of 1 *M* hydrochloric acid. The sample was centrifuged once more and the sediment was air dried after the supernatant had been discarded. The sediment was dissolved in 200 μl of BCA test reagent and, after incubation for 30 min at room temperature, 150 μl were transferred into a flat-bottomed 96-well plate. Absorbance at 570 nm was measured in an ELISA reader. For calibration, bovine serum albumin was used in a range from 2 to 100 μg in the assay.

3. Results

3.1. General conditions for HIC of haemolysins

We attempted to purify three bacterial haemolysins by hydrophobic interaction chromatography. HIC demands binding of proteins at high salt concentrations (usually ammonium sulphate) to increase the hydrophobic character of the protein. First, the ammonium sulphate concentration at which the haemolysins begin to precipitate was determined. Aliquots of crude culture supernatants containing $2.1 \cdot 10^3$ U/ml of listeriolysin O (60 μg/ml of protein), $1.3 \cdot 10^3$ U/ml of alpha-toxin (140 μg/ml of protein) or 55 U/ml of streptolysin O were subjected to concentrations of ammonium sulphate ranging from 20% to 50% saturation in 5% steps in PBS. After incubation for 1 h on ice, the samples were centrifuged and their haemolytic activities in the supernatants were assayed. Listeriolysin O, alpha-toxin and streptolysin O were precipitated at 30%, 50% and 40% saturation of ammonium sulphate, respectively.

In order to identify suitable HIC material for each of the haemolysins, binding experiments were performed in a batch assay using three HIC resins with increasing hydrophobicity from butyl- to phenyl- to octyl-Sepharose. Listeriolysin O ($2.4 \cdot 10^4$ U/ml, 830 μg/ml of protein) or alpha-toxin ($9.5 \cdot 10^3$ U/ml, 1.14 mg/ml of protein) after ammonium sulphate precipitation and the dissolved streptolysin O (55 U/ml) were adjusted to 27%, 45% or 35% ammonium sulphate saturation in PBS, respectively, the highest concentrations of ammonium sulphate at which no precipitation occurred. To 200 μl of packed and equilibrated HIC resins, 200 μl of haemolysin were mixed in microcentrifuge tubes. After incubation on ice for 30 min and centrifugation, the residual haemolytic activities in supernatants were determined. Each haemolysin was treated similarly with HIC resins without ammonium sulphate in order to determine whether the toxins were bound already at low ionic strength. Octyl- and phenyl-Sepharose bound all three haemolysins in the absence of ammonium sulphate (Table 1); therefore, these media were too hydrophobic to be used in purification. In con-

Table 1
Purification of the haemolysins on HIC columns

Haemolysin[a]	HIC medium	Binding condition (% ammonium sulphate)[b]	Elution from HIC column (% ammonium sulphate)[c]	Recovery (%)[d]	Specific activity (U/mg protein)
Listeriolysin O	Octyl-Sepharose CL-4B	0	NA[e]		
	Phenyl-Sepharose CL-4B	0	NA		
	Phenyl-Sepharose low binding	27	0		
	Butyl-Sepharose	27	13–0	75–85	440 000
	Alkyl-Superose	27	10–3	12	ND[f]
	Methyl-HIC cartridge	27	10–0	6	ND
Alpha-toxin	Octyl-Sepharose CL-4B	0	NA		
	Phenyl-Sepharose CL-4B	0	NA		
	Phenyl-Sepharose low binding	45	0		
	Butyl-Sepharose	45	22–5	20	17 000
	Alkyl-Superose	45	34–30	70–90	29 000
	Methyl-HIC-cartridge	45	25–17	45–50	28 000
Streptolysin O	Octyl-Sepharose CL-4B	0	NA		
	Phenyl-Sepharose CL-4B	0	NA		
	Butyl-Sepharose	35	8–0	38	ND
	Alkyl-Superose	35	11–0	5	ND
	Methyl-HIC cartridge	35	23–12	32	ND

[a] Starting material contained $2.4 \cdot 10^4$ U/ml of listeriolysin O ($2.9 \cdot 10^4$ U/mg of protein), $9.5 \cdot 10^3$ U/ml of alpha-toxin ($8.3 \cdot 10^3$ U/mg of protein) and 55 U/ml of streptolysin O.
[b] Binding at the indicated concentration (% saturation) of ammonium sulphate. Further conditions are described in the text.
[c] The range of concentration (% saturation of ammonium sulphate) of those fractions within a decreasing linear gradient of ammonium sulphate with haemolytic activity is given.
[d] Total haemolytic activity recovered related to the activity loaded on to the column.
[e] NA = not applicable.
[f] ND = not determined.

trast, butyl-Sepharose did not bind any of the toxins at low ionic strength, but completely bound listeriolysin O, alpha-toxin and streptolysin O at 27%, 45% or 35% ammonium sulphate saturation, respectively. Conversely, all three haemolysins could be eluted with decreasing concentration of ammonium sulphate (Table 1).

3.2. *Separation of the three haemolysins on weak HIC columns*

Butyl-Sepharose, alkyl-Superose and Econo-Pac methyl-HIC cartridges exhibit weak hydrophobicity. The three haemolysins were loaded on to each of these columns and eluted with a decreasing linear gradient of ammonium sulphate (Table 1, Fig. 1). All fractions were subjected to the haemolytic assay, positive fractions were pooled and recoveries were determined quantitatively by serial dilutions of the materials loaded on to the columns and also of the pooled fractions (Table 1). In terms of recovery, listeriolysin O was best purified on butyl-Sepharose (recovery 75–85%, purification factor 15); alpha-toxin was best purified on alkyl-Superose (recovery 70–90%, purification factor 3.5) and acceptably well on Econo-Pac methyl-HIC cartridges (recovery 45–50%, purification factor 3.4). For streptolysin O, butyl-Sepharose proved to be a suitable HIC column, with a recovery of 38%; owing to the small amounts of protein in the purchased material, the specific activity and the purification factor could not be determined reliably. Analysis of pooled haemolytic fractions by SDS-PAGE indicated

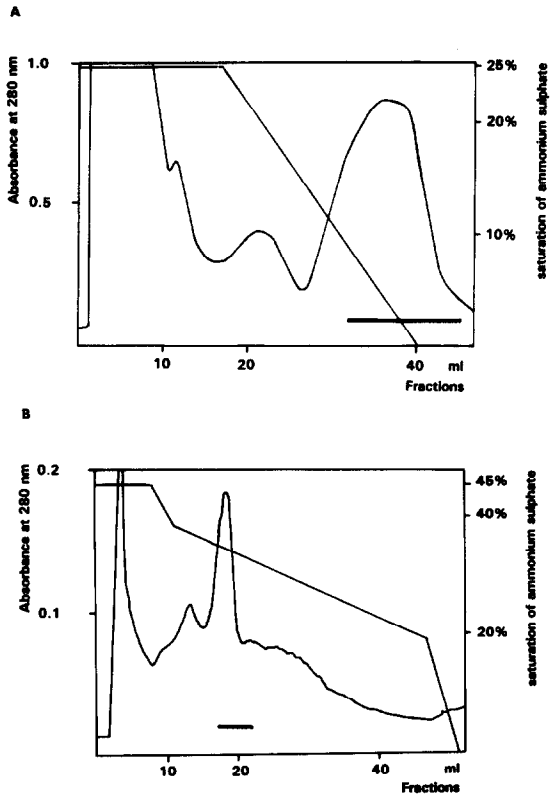

Fig. 1. Chromatography of (A) listeriolysin O on butyl-Sepharose and (B) alpha-toxin on alkyl-Superose. The decreasing gradient indicates decreasing saturation of ammonium sulphate from 25% [(A) listeriolysin O] or 45% [(B) alpha-toxin] to 0%. Haemolytic fractions are indicated with bars. A 10-ml volume (20 mg of protein) of crude listeriolysin O after precipitation with ammonium sulphate were loaded on to a 5-ml column of butyl-Sepharose and 1 ml (1.1 mg of protein) of crude alpha-toxin after precipitation with ammonium sulphate was loaded on to an alkyl-Superose HR5/5 column.

that alpha-toxin was purified to a single band (Fig. 2, lane 7). The major contaminants from the culture supernatants containing listeriolysin O had been removed by this single purification step (Fig. 2, lane 3). The identity of listeriolysin O was confirmed immunologically by western blotting with the listeriolysin O-specific mAb B8B20-3-2 [22] (Fig. 2, lane 5).

In order to evaluate the capacity of butyl-Sepharose for listeriolysin O, alkyl-Superose and Econo-Pac methyl-HIC cartridge for alpha-toxin, bacterial culture supernatants adjusted to the

Fig. 2. SDS-PAGE of the cytolsin fractions in 12.5% polyacrylamide gels revealed by silver staining (except lane 5). Molecular mass markers are on lane 1. Listeriolysin O fractions are on lanes 2–5: 2 = culture supernatant after precipitation with ammonium sulphate (4.8 μg); 3 = after HIC (2.8 μg); 4 = after gel filtration (2.3 μg); 5 = after HIC immunologically detected with antilisteriolysin O mAb B8B20-3-2 after western blotting. Alpha-toxin fractions are on lanes 6 and 7: 6 = culture supernatant after precipitation with ammonium sulphate (18 μg); 7 = after HIC (1.6 μg). A control with sample buffer only is on lane 8. kDa = kilodalton.

appropriate ammonium sulphate concentration were pumped on to 1-ml columns (butyl-Sepharose from the HIC test kit and alkyl-Superose HR5/5) and 5-ml columns (Econo-Pac methyl-HIC cartridge) until haemolytic activities appeared in the effluent. The capacity of butyl-Sepharose for listeriolysin O was $4.6 \cdot 10^5$ U (or 1.8 mg of protein) per milliliter of resin material; this amount was contained in 200 ml of culture supernatant with $2.8 \cdot 10^3$ U/ml and 85 μg/ml of protein. The capacity of alkyl-Superose for alpha-toxin was $6.9 \cdot 10^4$ U per millilitre of resin, corresponding to 2.6 mg of toxin; this amount of toxin was contained in 110 ml of culture supernatant with 900 U/ml and 110 μg/ml of protein. A 5-ml volume of Econo-Pac methyl-HIC medium bound $5.4 \cdot 10^4$ U (or 2.4 mg of protein) from 125 ml of culture supernatant.

3.3. Further purification of listeriolysin O

For further purification of listeriolysin O we intended to use anion-exchange chromatography, in analogy with a published method [25] which used DEAE-Sephacel with 25 m*M* Tris–hydrochloric acid buffer (pH 8.0). When applied to the Mono Q HR5/5 anion-exchange column, listeriolysin O eluted between 0.25 and 0.27 *M* sodium chloride in this buffer. We obtained only

Table 2
Purification of listeriolysin O

Purification step	Total activity (10^5 U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)
Culture supernatant[a]	7.2	21	34 000	–
Ammonium sulphate precipitation	5.8	20	29 000	81
Butyl-Sepharose HIC	4.9	1.1	440 000	68
Superose 12 HR10/30 gel filtration	1.6	0.26	620 000	22

[a] Starting material was 350 ml of culture supernatant containing 2100 U/ml of listeriolysin O and 60 μg/ml of protein.

a 5% recovery but we cannot compare this result because the recovery was not reported by the previous authors. Listeriolysin O bound to the Mono S HR5/5 cation-exchange column and eluted between 0.1 and 0.11 *M* sodium chloride in 50 m*M* sodium malonate buffer (pH 6.0 or pH 7.5), each with a recovery of around 15%. However, gel filtration in PBS (pH 6.0) on a Superose 12 HR 10/30 column resulted in pure toxin and a higher recovery (32%). On SDS-PAGE, a single band was visible (Fig. 2, lane 4). In combination with the previous HIC, listeriolysin O could be purified in a two-step process to a specific activity of $6.2 \cdot 10^5$ U/mg and a total recovery of 22% (Table 2).

4. Discussion

We have described an efficient method for the purification of representative bacterial haemolysins by HIC, which is probably also applicable for other cytolysins. Both HIC resins and ion exchangers allow purification and concentration in a single step and both are appealing first-step methods for the purification and concentration of proteins from culture supernatants. Ion exchangers are particularly useful owing to (i) the low cost of further additives and the technical possibilities of floating systems where bacterial suspensions can be pumped directly through specially designed columns such as Streamline (Pharmacia), thereby avoiding the separation of culture supernatants from bacteria by centrifugation. Difficulties in using ion exchangers can arise from osmolarity of the culture medium hindering binding of the proteins of interest. HIC resins can be used especially in media of high osmolarity, but require further additives such as ammonium sulphate. In this instance the lowest possible concentration has to be tested if cost becomes a factor of consideration. HIC, however, can be very efficient for the separation of proteins of interest from contaminants. Weak HIC resins proved particularly useful purification media for crude material if the proteins of interest were bound. They allow efficient purification because only a few proteins bind to weak HIC media. In previous work we purified recombinant heat shock protein of the M_r 60 000 family from *Mycobacterium bovis* BCG from crude lysates of the host *Escherichia coli* on alkyl-Superose with high efficiency [26]. This recombinant protein could be purified in a single step from almost all contaminating proteins that did not bind to the weak HIC column. At the same time, among all the separation methods tested, only alkyl-Superose enabled us to separate this protein from a truncated heat shock protein of which the M_r was 3000 lower. Cytolysins, in contrast to most other cellular proteins, interact with membranes, *i.e.*, with hydrophobic areas. Therefore, it is reasonable to consider HIC with columns of weak hydrophobicity for the separation of cytolysins from most proteins.

According to the principles controlling HIC [14], experiments to evaluate the potential of HIC for the purification of proteins should

address the following questions: (i) at which concentration of ammonium sulphate does the protein start to become salted out; (ii) which HIC columns contain too strong hydrophobic ligands, thus binding the proteins already in the absence of antichaotropic agents; (iii) which HIC columns contain too weak hydrophobic ligands, preventing binding of the protein at the highest possible concentrations of ammonium sulphate; (iv) what are the conditions for optimum chromatography in terms of recovery and purity on those HIC columns which allow binding of the protein at high concentrations of ammonium sulphate and desorption with decreasing concentrations of ammonium sulphate?

Concentrations of ammonium sulphate at which listeriolysin O, streptolysin O and alpha-toxin still remained in solution allowed their binding to HIC media with ligands of weak hydrophobicity such as butyl-Sepharose and alkyl-Superose. HIC media of medium hydrophobicity such as phenyl-Sepharose possessed too strong binding properties for these haemolysins. Because we obtained different yields using different chromatographic resins of weak hydrophobicity, the optimum HIC resin had to be identified for each cytolysin individually. Oligomerization of varying degrees on different HIC media could provide an explanation for these different recoveries.

Comparing HIC with other separation methods, for listeriolysin O HIC was more efficient in terms of recovery than chromatography with thiol-activated Sepharose, the recoveries being 80% and 11% [9], respectively. Together with gel filtration, a specific activity of $6.2 \cdot 10^5$ U/mg was obtained, comparable to published specific activities of $10 \cdot 10^5$ U/mg, and our method resulted in a higher total recovery. For alpha-toxin, the recovery from alkyl-Superose was high (>80%) and the specific activity of $2.9 \cdot 10^4$ U/mg was comparable to published specific activities of the purified toxin, *e.g.*, $3.1 \cdot 10^4$ U/mg [12] and $2 \cdot 10^4$–$5 \cdot 10^4$ U/mg [27]. The high separation power of the HIC columns used can be perceived visually from SDS-PAGE (Fig. 2). In crude culture supernatants the haemolysins are the major protein. By comparing the protein pattern on SDS-PAGE before and after HIC, it is obvious that the haemolysins are separated from a large pool of different proteins not bound by the HIC columns. The binding capacity of the HIC columns used allowed the purification of 1 l of culture supernatant of listeriolysin O with a 5-ml column of butyl-Sepharose and of alpha-toxin with a 10-ml column of alkyl-Superose or a 40-ml methyl-HIC cartridge.

Comparing our results using HIC for listeriolysin O, streptolysin O and alpha-toxin with those of other investigators who applied HIC successfully for the purification of bacterial haemolysins [15–19], we conclude that in general high recoveries and purities can be obtained with weakly hydrophobic HIC columns. In addition to covalent chromatography on thiolated matrices as a general method for the separation of thiol-activated toxins, HIC could provide another approach of high potential for the purification of cytolytic proteins.

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