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Removal of endotoxins by affinity sorbents

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Abstract

Histidine, histamine and polymyxin B affinity sorbents were employed for the removal of *Escherichia coli*-derived endotoxins. Their effectiveness was compared with those of poly-L-lysine-Sepharose and DEAE-Sepharose. All sorbents reduced the concentration of endotoxins from an *E. coli* culture filtrate to tolerable levels. However, their effectiveness was not higher than that of the anion exchanger, which displayed clearance rates of up to 15 000. Endotoxin removal from protein solutions depended on the net charge of the desired protein. Lysozyme as a model for positively charged proteins enhanced endotoxin removal. In contrast, only low initial contamination levels (<34 EU/ml) were reduced to tolerable levels from bovine serum albumin (BSA) as the negatively charged protein model owing to competition of BSA and endotoxins for adsorption sites. Hence also a low BSA recovery was observed after the treatment whereas the lysozyme recovery was almost 100%. At pH values below the isoelectric point of BSA, endotoxin removal was also more effective. The best conditions for the decontamination were found at neutral pH and low ionic strength (≤20 m*M* phosphate). Ionic forces between ligands and endotoxins are dominant at this ionic strength; hydrophobic interactions are not very effective. Hence the selectivities of all sorbents towards endotoxins are not exceptionally high. DEAE-anion exchangers are the most suitable sorbents for the removal of endotoxins from solutions accommodating positively charged proteins owing to their low cost and high capacity. Poly-L-lysine-Sepharose was most effective for the removal of small amounts of endotoxins from solutions of negatively charged proteins. The "affinity ligands" histamine, histidine and polymyxin B were effective for the removal of endotoxins from *E. coli* filtrate; however, their effectiveness decreased dramatically in the presence of BSA and it was lower than for poly-L-lysine- and DEAE-Sepharose in the presence of lysozyme.

1. Introduction

Pyrogens are lipopolysaccharides from the cell wall of Gram-negative bacteria or other constituents, such as polypeptides or peptidoglycans of viruses and fungi. Lipopolysaccharides from *Escherichia coli*, which are more commonly termed endotoxins, constitute one of the major problems in the formulation of pharmaceutical products. They produce fever in man or mammals after intravenous application at concentrations less than 1 ng/ml [1].

The exploitation of *E. coli* as host for the production of pharmaceutical proteins embraces the risk of contamination with endotoxins. Hence it is essential to reduce the endotoxin level to tolerable concentrations during downstream processing. Common purification trains, accommodating several chromatographic sorbents of different composition, provide acceptable clearance rates in many cases [2–4]. On the other hand, the clearance of endotoxins by just one step is difficult owing to the chemical

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composition of these pyrogens. Therefore, the introduction of endotoxins during the downstream process, as is observed after microbial contamination of chromatographic columns or equipment, is carefully avoided. If endotoxin contamination takes place despite these precautions, methods for their removal should be available. For practical reasons, efficiency, costs and high product recovery during this treatment are the most important aspects.

Methods for endotoxin removal, such as adsorption on activated carbon [5,6] or ion exchangers [7], display low selectivity, thus often leading to low product recoveries. Ultrafiltration [8,9] or size-exclusion chromatography require large differences in size between the product and contaminant; however, they are not effective even if the contaminant binds strongly to the target protein.

The application of affinity chromatographic sorbents based on histamine, histidine [10–13] and polymyxin B [14,15] ligands is described as a method to remove endotoxins from protein solutions without denaturation and loss of products. Clearance rates up to 10^4 are reported if *E. coli* culture filtrates or prepurified endotoxin solutions are employed under defined conditions. However, the clearance of endotoxins from protein solutions with such endotoxin-specific sorbents is intended to be employed in the case of an emergency. Hence this process can be considered as a polishing step where ideally the composition of the protein solution, as settled after product formulation, can be varied over a wide range. This process should be effective in order to reduce endotoxins to the picogram level, should not adsorb the product and should not be time consuming. Therefore, a sorbent with exceptional specificity for endotoxins is demanded, which should also display fast adsorption kinetics as well.

Endotoxins are mainly composed of lipid moieties in addition to carbohydrate sites and phosphate groups. Hence both hydrophobic and ionic forces are possible interactions with other molecules [1]. Endotoxin-specific sorbents composed of histidine, histamine and polymyxin B also display both ionic and hydrophobic sites (Fig. 1: characteristic data are compiled in Table 1). Histidine is immobilized through a 1,6-diaminohexane spacer; commercial histamine-immobilized Sepharose accommodates sixteen atoms. Polymyxin B is immobilized without a spacer on CNBr-activated Sepharose, but it consists of several hydrophobic amino acids. It is concluded in the literature that the synergistic effects of hydrophobic and ionic interactions are the major reason for the selectivity of these sorbents for endotoxins. It is realistic that sorbents consisting of similar characteristics may also display selectivities towards endotoxins. Thus, poly-L-lysine was employed as a further ligand, because it is also slightly hydrophobic due to the ϵ-aminobutyl functional group besides its mainly ionic properties.

This study was aimed at a comparison of the effectiveness of the three endotoxin-specific sorbents described above with poly-L-lysine-immobilized Sepharose 4B and DEAE-Sepharose, which can be considered as the most hydrophilic sorbent employed. It was of particular interest to compare clearance rates from an untreated *E. coli* culture filtrate with protein solutions of BSA and lysozyme and defined contamination levels. Hence cross-selectivities of these sorbents with BSA and lysozyme were also investigated. A further objective was the investigation of the influence of the ionic strength and pH on the removal of endotoxins from these protein solutions.

2. Experimental

2.1. Materials

Histamine- and polymyxin B-Sepharose CL-4B, poly-L-lysine-Sepharose 4B, DEAE-Sepharose CL-6B, L-histidine and 1,6-hexamethylenediamine were purchased from Sigma, (Munich, Germany), Coatest endotoxin (Limulus Amoebocyte Lysate test) from Chromogenix (Mölndal, Sweden), bovine serum albumin (BSA) from Serva (Heidelberg, Germany) and Sepharose CL-4B, lysozyme (chicken egg) and pyrogen-free water from Fluka (Neu-

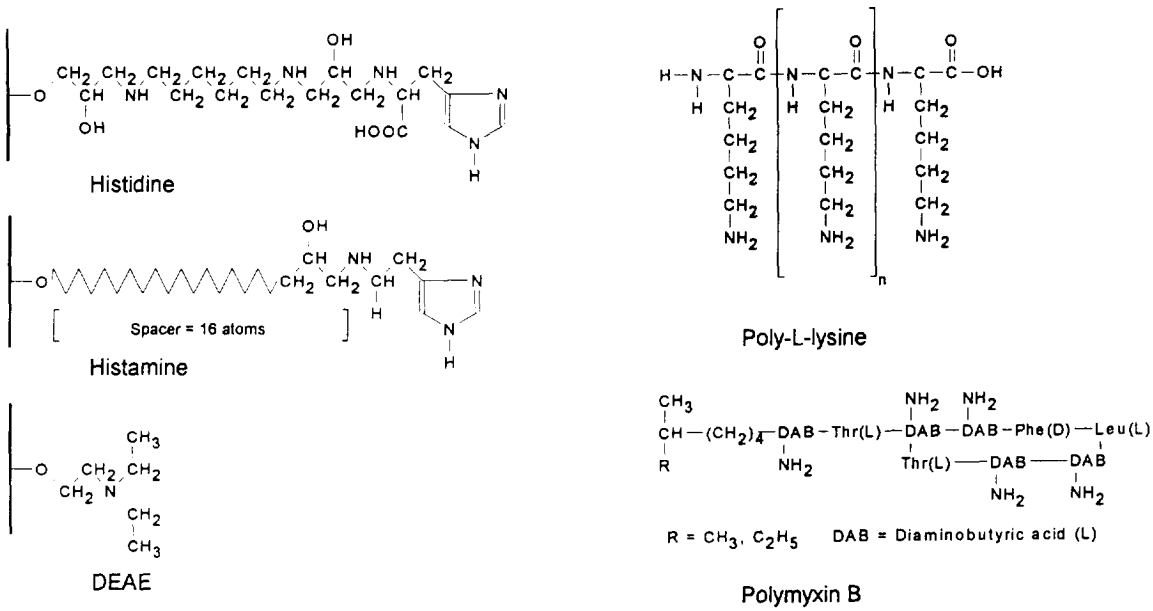

Fig. 1. Structures of endotoxin-specific ligands employed in this study. All ligands contain both ionic and hydrophobic functional groups; DEAE can be considered as least hydrophobic.

Ulm, Germany). All other chemicals were of analytical-reagent grade.

2.2. Preparation of *E. coli* culture filtrate

E. coli cells were grown in minimum medium according to DIN 53739 with glucose (10 g/l) at 310 K overnight. A sterile culture filtrate from this culture was obtained by centrifugation (2 × 7000 *g*, 20 min) using a Suprafuge 22 centrifuge (Heraeus, Osterode, Germany) and filtration (pore size 0.22 μm). Endotoxin concentrations varied on average around 70 000 EU/ml and protein concentrations of 0.1 mg/ml.

2.3. Preparation of immobilized histidine

Epibromohydrin-activated Sepharose CL-4B was prepared as described by Hochuli et al. [16].

Table 1
Some properties of protein models and ligands employed as endotoxin-specific sorbents

Protein/ligand	p*K*	p*I*
BSA	-	4.8
Lysozyme	-	11.2
Histidine and histamine	6.0 (imidazole)	7.6
	>9 (α-amino group)	
	>10.5 (NH group of diaminohexyl spacer)	-
Polymyxin B	>9 (α-amino group of DAB)	
	~10.5 (δ-amino group of DAB)	
Poly-L-lysine	10.8 (ε-amino group of monomer)	9.7 (monomer)
DEAE	9–9.5	

Immobilization of histidine was either performed directly on activated Sepharose at pH 12 without incorporation of a spacer arm or by the procedure described by Minobe et al. [11]. Briefly, 10 ml of epibromohydrin-activated Sepharose CL-4B were suspended in 80 ml of water and adjusted to pH 11 with sodium hydroxide, then 2.15 m*M* 1,6-hexamethylenediamine was added and the mixture was stirred at 333 K for 2 h to yield aminohexyl-Sepharose (AH-Sepharose). The collected AH-Sepharose was washed with water and suspended in 4 *M* NaOH. Epibromohydrin was added and the mixture stirred at 333 K for 10 min. The activated AH-Sepharose CL-4B was collected and washed with water. A 1-g amount of L-histidine was dissolved in water, adjusted to pH 12 with concentrated sodium hydroxide and then heated to 253 K. Finally, activated AH-Sepharose CL-4B was added to the solution and stirred at 253 K for 40 min. The histidine-Sepharose was collected and washed with water.

2.4. Adsorption of endotoxins

An FPLC 500 system from Pharmacia (Uppsala, Sweden) was used for all adsorption experiments. The various sorbents were packed in standard FPLC columns of 25–30 mm bed height and 5 mm I.D. The endotoxin source was derived from the *E. coli* culture filtrate. After equilibration, a sample containing various concentrations of endotoxins was loaded on the column at a flow-rate of 0.4 ml/min for 80 min at 293 K. Phosphate buffer (20 m*M*) was used at pH 3.8, 7.0 and 8.5; one experiment was carried out at increased ionic strength (100 m*M* phosphate, pH 7). The concentrations of endotoxins and proteins were measured in the column effluent.

All resins were regenerated by passing 25 column bed volumes of 0.2 *M* NaOH containing 20% ethanol through the column, followed by 25 bed volumes of 1.5 *M* NaOH, as recommended for the desorption of endotoxins from the histidine sorbent [11]. Potassium phosphate buffers of 20 or 100 m*M* were used as equilibration buffer at various pH values.

2.5. Endotoxin assay

Endotoxins were assayed by the chromogenic Limulus Amoebocyte Lysate (LAL) test. In order to minimize the analysis costs, only half the amounts recommended in the specification was used in all experiments. The accuracy of the measurements was sufficient to allow conclusions to be drawn. However, for clinical applications this is not recommended.

2.6. Protein assay

Protein concentrations were assayed according to the method of Lowry et al. [17], with BSA and lysozyme as standards.

2.7. Determination of adsorption isotherms with BSA

Frontal analysis was conducted with the FPLC system to determine the adsorption isotherms of the various resins with BSA. Different concentrations of BSA were loaded on the columns at 20 m*M* phosphate (pH 7.0); a breakthrough curve was recorded using a C-R3A integrator (Shimadzu, Kyoto, Japan) and the breakthrough time was determined as the median bisector [18]. The amount of adsorbed BSA (q^*) was determined from the breakthrough time and the initial concentration of BSA; it was plotted versus the concentration of free BSA (c^*). Apparent dissociation constants and saturation capacities were calculated assuming single-site interaction of ligand and solute. In the case of Langmuir-type isotherms a double reciprocal plot of $1/q^*$ against $1/c^*$ gave q_m from the intercept on the ordinate and K_D from the slope of the linear plot [19,20].

2.8. Determination of adsorption isotherms with endotoxins

The adsorption isotherms of the various resins with endotoxins were determined by performing

batch experiments. Portions of 450 μl of diluted culture filtrate with various endotoxin concentrations was mixed with 50 μl of resin suspension and shaken overnight. A small amount of resin was required to account for the relatively low endotoxin concentration in the culture filtrate which corresponded to a concentration of 7 μg/ml (70 000 EU/ml) at the maximum. The concentrations of endotoxins in the supernatant was measured and plotted versus the amount of adsorbed endotoxins.

3. Results

3.1. Removal of endotoxins from E. coli culture filtrate

Histidine sorbents obtained through direct immobilization of histidine on epibromohydrin-activated Sepharose 4B did not show a significant removal of endotoxins (data not shown). This is in accordance with results published by Minobe and co-workers [11,13], who concluded that synergistic interactions of positive charges at the diaminohexane spacer and the histidine functional group cause strong interactions with endotoxins. Consequently, histidine was immobilized by a diaminohexane spacer in all further studies reported in this paper.

The effectiveness of histidine-, histamine-, polymyxin B-, poly-L-lysine- and DEAE-Sepharose in the removal of endotoxins was investigated by loading different dilutions of the *E. coli* culture filtrate containing 370, 1200 and 7170 EU/ml in 20 m*M* phosphate buffer (pH 7.0) on the columns. The results are summarized in Table 2. All sorbents reduced the concentration of endotoxins from diluted *E. coli* culture filtrates to a level below the threshold pyrogenic dose of *E. coli* LPS (1.0 ng/kg, corresponding to ca. 10 EU/kg) at a contamination of 1200 EU/ml at the maximum. The best results were obtained with histidine and polymyxin B with clearance rates up to 1000 and 2180, respectively. The concentration of endotoxins in the column effluent increased, depending on the starting concentration, except with polymyxin B, which displayed similar concentrations at the two lower initial concentrations.

Higher initial concentrations led to insufficient removal of endotoxins for parenteral drugs (endotoxin limit 5 EU/ml), without exception. With these concentrations the clearance rates also decreased significantly. The best results were obtained with the histidine sorbent. Using culture filtrate, this sorbent is the most effective compared with the others under investigation. In contrast to published results, where clearance rates of up to 10^4 were reported using prepurified endotoxin sources in defined solutions [11], the endotoxin source in this study also accommodated host-specific proteins (0.1 mg/ml) and metabolic products of undefined composition in a heterogeneous medium. However, the decrease in clearance rates at the highest endotoxin level is probably not caused by the ionic

Table 2
Removal of endotoxins from *E. coli* culture filtrates

Ligand	A	B	C	Lit.
Histamine	0.9 (410)	2.3 (520)	196 (36)	10^4 [10,11]
Histidine	0.55 (670)	1.2 (1000)	24 (290)	200–10^4 [10,11]
Polymyxin B	0.8 (460)	0.55 (2180)	110 (65)	650–10^4 [14,15]
Poly-L-lysine	1.3 (280)	2.7 (440)	48 (150)	–
DEAE	1.3 (280)	2.9 (410)	105 (68)	–

Data are endotoxin concentrations in the column effluent in EU/ml, with clearance rates in parentheses. Initial concentrations of endotoxin, (A) 370, (B) 1200 and (C) 7170 EU/ml, each diluted in 20 m*M* potassium phosphate buffer (pH 7.0).

strength, since even the highest endotoxin concentration was diluted tenfold with the 20 m*M* phosphate buffer.

3.2. Removal of endotoxins from lysozyme and BSA solutions at pH 7.0

The effectiveness of the sorbents in the removal of endotoxin contaminations was investigated by using lysozyme and BSA as positively and negatively charged model proteins, respectively. Solutions containing 1 mg/ml of commercial lysozyme in 20 m*M* phosphate buffer (pH 7.0) were contaminated with 10.9 EU/ml, as determined with the LAL assay. Endotoxin levels as high as 1500 EU/ml were obtained after addition of endotoxins from the culture filtrate. Both solutions were pumped on to the columns under the conditions described above. BSA solutions [also 1 mg/ml in 20 m*M* phosphate buffer (pH 7.0)] contaminated with 33.7 and 1490 EU/ml from the *E. coli* culture filtrate were used for the experiments. Clearance rates and protein recoveries are given in Table 3 and Fig. 2, respectively.

The removal of endotoxins from the lysozyme solution was most successfully performed with DEAE and poly-L-lysine. Both sorbents reduced endotoxin contaminations to the lower detection limit of the LAL assay. With the DEAE anion exchanger the highest clearance-rate (15 000) was measured; such a high clearance was not exceeded in this study. Although lysozyme is positively charged and association with negatively charged endotoxins is likely, the clearance rates in the presence of lysozyme were more than an order of magnitude higher than in the absence of this protein. In contrast, the clearance rates of histamine, histidine and polymyxin B sorbents were reduced in the presence of lysozyme, indicating a deviate interaction mechanism. The clearance rates increased with increasing initial endotoxin concentration with all sorbents.

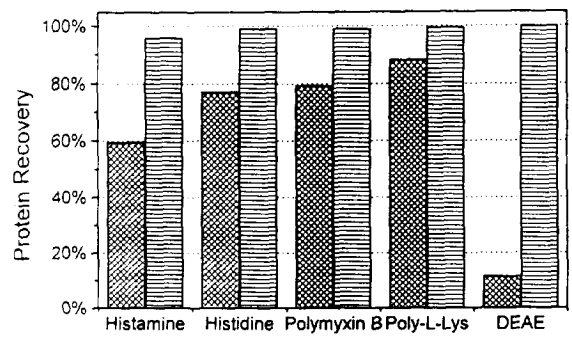

Fig. 2. Mass recovery of BSA and lysozyme (cross- and horizontal-hatched, respectively) after decontamination. Proteins dissolved in diluted culture filtrate (1 mg/ml) in 20 m*M* potassium phosphate buffer (pH 7.0); initial contaminations 1490 EU/ml with BSA and 1500 EU/ml with lysozyme.

On average, 1% lysozyme adsorption was observed on the sorbents (Fig. 2), except for histidine, which displayed about 5%; 1% adsorption is insignificant considering the experimental error during protein determination,

Table 3
Removal of endotoxins from protein solutions

Ligand	BSA		Lysozyme	
	A	B	C	D
Histamine	0.76 (44)	*29.3* (50)	0.04 (272)	4.8 (312)
Histidine	2.15 (15)	*21.6* (68)	0.65 (17)	5.4 (278)
Polymyxin B	2.4 (14)	*30* (50)	0.33 (33)	2.2 (680)
Poly-L-lysine	1.0 (34)	*24.0* (62)	0.03 (310)	0.21 (7,140)
DEAE	0.21 (160)	*21.9* (68)	0.08 (136)	0.1 (15,000)

Data are endotoxin concentrations in the column effluent in EU/ml (values in italic type exceed the threshold level), with clearance rates in parentheses. Initial concentration of endotoxins, (A) 33.7, (B) 1490, (C) 10.9 and (D) 1500 EU/ml, each diluted in 20 m*M* potassium phosphate buffer (pH 7.0); solutions contained 1 mg/ml of either BSA or lysozyme.

but 5% indicates the presence of interactions between lysozyme and the histidine ligand. Endotoxins bound with a lower affinity to all sorbents in the presence of BSA, resulting in lower clearance rates (50–70) with all sorbents at high endotoxin contamination (Table 3). Further, the recovery of BSA was only 10% on the DEAE anion exchanger (on the time-scale of the experiment) and was also unsuitable with the other sorbents. The highest recovery (88%) was found on the poly-L-lysine-sorbent (Fig. 2) under these chromatographic conditions.

Both results indicated that the high clearance rates obtained for histidine or polymyxin B sorbents and culture filtrates or prepurified endotoxin solutions are not transferable to more realistic conditions where proteins are present. In the presence of positively charged lysozyme, the clearance with these sorbents is less effective than with DEAE or poly-L-lysine. In the presence of negatively charged BSA, the clearance of all sorbents is comparable. Protein–endotoxin interactions does not seem to control the clearance effectiveness of the sorbents, since then lysozyme would have carried the endotoxins through the columns, being repelled itself from positively charged ligands. Instead, the competing interactions of target proteins and endotoxins on the stationary phase appears to be of most importance.

3.3. Effect of ionic strength

Increasing the ionic strength from 20 to 100 m*M* phosphate in the presence of BSA or lysozyme resulted in a decrease in the clearance rates with all sorbents (Table 4). In comparison with the experiments in 20 m*M* phosphate, the clearance rates with BSA solutions decreased at least eightfold, while those from the lysozyme solutions decreased up to 1900-fold. The DEAE- and poly-L-lysine-functional sorbents were most affected. This indicates that adsorption of endotoxins on these sorbents is mainly controlled by ionic interactions. Although the other sorbents removed ca. 95% of endotoxins, the maximum threshold level was exceeded at least tenfold. Hence a low ionic strength is a fundamental requirement to allow effective endotoxin removal. The dependence of the interaction with histidine on ionic strength is in agreement with published results [11,13]. The strongest interactions were reported at ionic strengths corresponding to 20–50 m*M* NaCl and above 3 *M* NaCl with this ligand. At such high NaCl concentrations, hydrophobic interactions become dominant.

Table 4
Effect of increase in ionic concentration on the removal of endotoxins

Ligand	BSA	Lysozyme
Histamine	>150 (>8)	89 (13.5)
Histidine	>150 (>8)	114.5 (10.5)
Polymyxin B	>150 (>8)	58.6 (20.5)
Poly-l-lysine	>150 (>8)	130 (9.0)
DEAE	>150 (>8)	46.2 (8.0)

Data are endotoxin concentrations in the column effluent in EU/ml, with clearance rates in parentheses. Initial concentration of endotoxins: BSA 1190 and lysozyme 1200 EU/ml, each diluted in 100 m*M* potassium phosphate buffer (pH 7.0); solutions contained 1 mg/ml of either BSA or lysozyme.

With 100 m*M* phosphate (pH 7), the recovery of lysozyme decreased slightly on all sorbents, whereas the recovery of BSA increased significantly (data not shown). These results reflect the decrease in ion-exchange interactions of BSA with the sorbents, which can be directly linked to the higher ionic strength; lysozyme is less repelled and presumably interacts with hydrophobic moieties.

3.4. Effect of pH

Three series of experiments were performed to investigate the effect of pH on the removal of endotoxins (Table 5). At pH 3.8, the clearance rates on lysozyme solutions decreased slightly, whereas the clearance rates on BSA solutions increased significantly in comparison with neutral pH. The removal of endotoxins at pH 8.5 from BSA and lysozyme solutions was not effective.

The recovery of BSA decreased with increase

Table 5
Effect of pH on removal of endotoxins

Ligand	BSA			Lysozyme		
	pH 3.8	pH 7.0	pH 8.5	pH 3.8	pH 7.0	pH 8.5
Histamine	*10.3*	*29.3*	*>200*	*8.3*	4.8	*>200*
Histidine	*9.1*	*21.6*	*>200*	*7.6*	5.4	*>200*
Polymyxin B	*9.6*	*30*	*>200*	*10.3*	2.2	*>200*
Poly-L-lysine	*6.7*	*24*	*>200*	*6.2*	0.21	*>200*
DEAE	*8.3*	*21.9*	*>200*	*7.4*	0.1	*>200*

Initial concentration of endotoxins in BSA solution: pH 3.8, 1200 EU/ml; pH 7.0, 1490 EU/ml; pH 8.5, 3100 EU/ml. Initial concentration of endotoxins in lysozyme solution: pH 3.8, 1210 EU/ml; pH 7.0, 1500 EU/ml; pH 8.5, 3110 EU/ml. *E. coli* culture filtrate in 20 m*M* potassium phosphate buffer (pH 7.0); protein concentration, 1 mg/ml. Values in italic type exceed the threshold level.

in pH. The recovery of lysozyme remained almost constant at pH 3.8 and 7.0, but decreased at pH 8.5 (Fig. 3).

Both endotoxin removal and protein recoveries reflect charge deviations of proteins and ligands at the different pH values employed. Whereas the net positive charge of lysozyme remains between pH 3.8 and 8.5, BSA displays positive charge at pH 3.8 and negative charge at pH 7.0 and 8.5, being governed by its isoelectric point (p*I* = 4.8). The different ligands also display charge transformations depending on pH and electron densities at their nitrogen atoms.

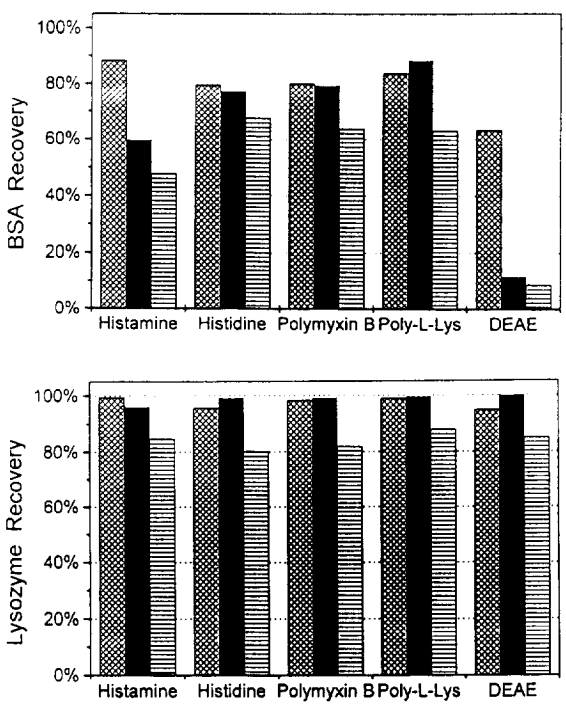

Fig. 3. Mass recovery of BSA and lysozyme at different pHs after decontamination. Proteins dissolved in diluted culture filtrate (1 mg/ml) in 20 m*M* potassium phosphate buffer; initial contaminations in BSA solution at pH 3.8 1200 EU/ml, pH 7.0 1490 EU/ml and pH 8.5 3100 EU/ml, and in lysozyme solution at pH 3.8 1210 EU/ml, pH 7.0 1500 EU/ml and pH 8.5 3110 EU/ml.

At pH 3.8, the charge density of negatively charged endotoxins is decreased owing to partial protonation of phosphorylated sugars at carbohydrate sites. As a consequence, endotoxin levels in the effluent and in the presence of lysozyme are higher than at pH 7, especially with anion exchangers.

At pH 8.5, the charge density at all sorbents is lower than at lower pH. For example, imidazole is not charged on histidine and histamine owing to a p*K* of 7 and also the α-amino group of histidine and the nitrogen atoms of the DEAE anion exchanger are partly uncharged owing to their p*K* (Table 1) and the Donnan effect. Therefore, removal of endotoxins is less effective. Repulsion of lysozyme is less pronounced; thus, hydrophobic interactions lead to a decrease in recovery. It is assumed that hydrophobic interaction is also the reason for the low recovery of BSA at elevated pH. In contrast, at pH 3.8 the positively charged BSA is not adsorbed by an anion-exchange mechanism, consequently leading to better recovery, especially on the DEAE anion exchanger. On other sorbents

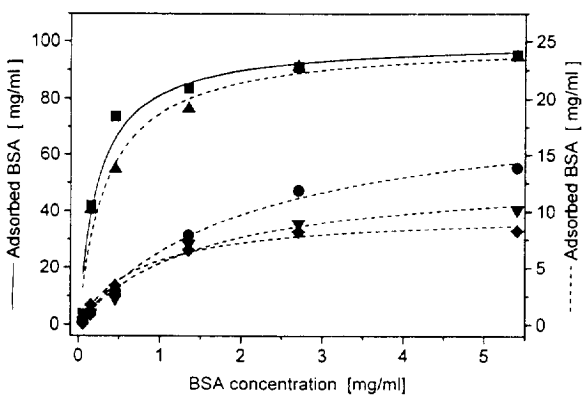

Fig. 4. Adsorption isotherms of BSA on different sorbents in 20 m*M* phosphate buffer (pH 7.0) at 298 K. ■ = DEAE-Sepharose CL-6B; ▲ = histamine-Sepharose 4B; ● = histidine-Sepharose CL-4B; ▼ = poly-L-lysine-Sepharose 4B; ◆ = polymyxin B-Sepharose 4B.

this increase in recovery is less pronounced, indicating the mixed-mode interactions of these sorbents, displaying both anion-exchange and hydrophobic moieties.

3.5. Adsorption of BSA on various sorbents

Owing to the low recovery of BSA at neutral and elevated pH, the thermodynamic parameters were investigated in more detail. Frontal chromatography on the various endotoxin-specific sorbents revealed Langmuir adsorption isotherms and, consequently, apparent dissociation constants were calculated (Fig. 4 and Table 6, respectively).

The adsorption isotherms displayed strong interactions of DEAE- and histamine-Sepharose with BSA, as indicated by the steep incline of these curves at low protein concentration (Fig. 4) and the low dissociation constants (Table 6). The other sorbents displayed lower interactions. It is assumed that ion-exchange interactions are the most dominant interactions with all sorbents above the p*I* of the protein. All sorbents accommodate at least one positive charge at pH 7, interacting with negatively charged proteins at the low ionic strength employed.

Table 6
Apparent dissociation constants, K_D, and capacities of various sorbents for BSA

Ligand	Capacity (mg/ml)	K_D (*M*)
Histamine	22.5	$3.0 \cdot 10^{-6}$
Histidine	33.1	$7.3 \cdot 10^{-5}$
Polymyxin B	9.6	$1.1 \cdot 10^{-5}$
Poly-L-lysine	15.9	$3.6 \cdot 10^{-5}$
DEAE	99.6	$3.1 \cdot 10^{-6}$

The highest capacity for BSA was found with the DEAE anion exchanger, which was not surprising considering its ion-exchange capacity; the lowest capacity was displayed by the polymyxin B sorbent. It can be assumed that the capacities found for BSA are related to the endotoxin binding capacity, as this contaminant is also negatively charged. Therefore, the polymyxin B sorbent probably displays the lowest capacity for endotoxins compared with all the other sorbents employed.

The high capacity of the anion exchanger for BSA also indicates that this sorbent is unsuitable for the removal of endotoxins from protein solutions accommodating such negatively charged proteins: the product would be lost. In this regard, polymyxin B or poly-L-lysine is more appropriate.

Interestingly, endotoxins were still adsorbed on all sorbents at pH 7 or 8.5, despite exhaustion of their capacity for BSA. Only slight perturbations of endotoxin concentrations in the column effluent were observed on comparing the results before, during and after the breakthrough of BSA (data not shown). Generally, an increase was observed during breakthrough; however, the extent differed with each sorbent. After the breakthrough a decline was measured, again with different extents. No conclusions on this behaviour can be drawn yet, since the fluctuations were only slightly larger than the experimental errors observed during endotoxin analysis and also the sequence of analysis points was not narrow enough on the time-scale of experiments to allow precise deductions. Nevertheless, one should expect a decrease in clearance rates during breakthrough of the product, especially if interactions between product and sorbents are likely.

3.6. Adsorption of endotoxins on various sorbents

The adsorption of endotoxins on the various sorbents was investigated in the batch mode. Owing to the principle of the measurement (tentative until the equilibrium concentration of endotoxin is finally assayed), the shape of some isotherms is ill-defined (Fig. 5). Apparent dissociation constants and capacities were not calculated owing to missing data points in the most interesting sections of the adsorption isotherms. Except for the histidine sorbent, the capacity was compared with published results and was found to be of the same order of magnitude (ca. 0.48 mg endotoxin/g of wet sorbent in this study, compared with 0.31 mg/g reported by Matsumae et al. [13]). This capacity is low compared with the capacity for proteins. The strength of the interaction seems to be of the same order of magnitude as was observed with BSA; hence no extremely strong interactions between endotoxins and endotoxin-specific sorbents should be expected as are observed with some exceptional affinity systems, such as the biotin–avidin system [21] or some antigen–antibody systems [22]. The adsorption isotherms of endotoxins on DEAE, polymyxin B and histidine sorbents belong to the high-affinity type, as is evident from the steep incline of the isotherms. The poly-L-lysine and histamine sorbents displayed lower interaction strengths.

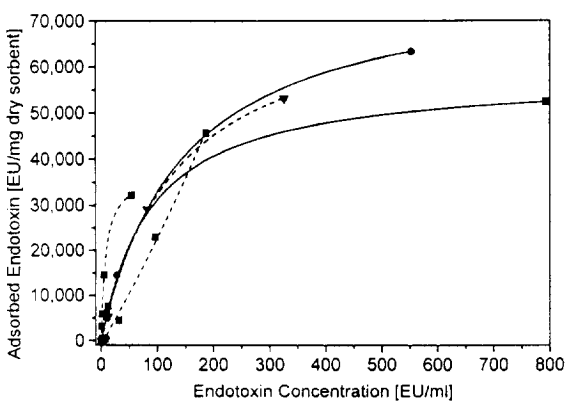

Fig. 5. Adsorption isotherms of endotoxins on sorbents in 20 m*M* phosphate buffer (pH 7.0) at 298 K. ■ = DEAE-Sepharose CL-6B; ▲ = histamine-Sepharose 4B; ● = histidine-Sepharose CL-4B; ▼ = poly-L-lysine-Sepharose 4B; ◆ = polymyxin B-Sepharose 4B.

4. Discussion

The results of this study clearly indicate that the removal of low and elevated concentrations of endotoxins from *E. coli* culture filtrate do not allow conclusions to be drawn about the effectiveness of the different sorbents in the presence of a protein. Although the clearance rates in the absence of a protein correspond well with results published from other groups (Table 2), better and worse clearance rates were observed in the presence of proteins with net positive and negative charges, respectively (Table 3).

Competing interactions with immobilized ligands of all the sorbents were observed in the presence of BSA; thus, none of the sorbents displayed exceptionally high selective properties for endotoxins. Only at the lowest endotoxin concentration employed (Table 3) were tolerable contamination levels achieved. At elevated endotoxin contaminations, unacceptable endotoxin levels were found. Owing to the interaction with negatively charged BSA, the recovery of this protein remained lower than with lysozyme. It is concluded that none of the ligands is generally applicable for endotoxin removal in the presence of negatively charged proteins. The best results were obtained with lysozyme as a protein model for positively charged proteins. With this protein, the clearance rates were even enhanced compared with the culture filtrate. Lysozyme is repelled from positively charged surfaces, which is why the lysozyme recovery was high at pH 7. It is most likely that endotoxins are associated with lysozyme at the low ionic strength utilized. Further, the interaction of lysozyme with cell surfaces of bacteria is known and a major function of this protein [23]. It seems that during passage of the lysozyme–endotoxin complex through the column bed, endotoxins dissociate from lysozyme and diffuse to the surface of the stationary phase, where they are strongly adsorbed owing to multi-point attach-

ment. Since lysozyme does not approach the positively charged surface very closely, it will not compete with endotoxins for adsorption sites. The enhanced clearance rates compared with the *E. coli* culture filtrate are probably a result of the absence of endotoxin agglomerates due lysozyme–endotoxin complexes which were formed earlier. This is confirmed by the deviating results observed at higher ionic strength (100 m*M* phosphate), where considerably decreased clearance rates were measured. Similar effects are observed with detergents, which enhance the dissociation of large endotoxin agglomerates into monomers [1,14]. At higher ionic strength, lysozyme–endotoxin complexes are absent and endotoxin agglomerates will form. Probably the decrease in lysozyme recovery is caused by hydrophobic interactions which also seem to interplay at elevated pH.

A low ionic strength of the applied protein solution is most recommended with all sorbents. This indicates that the adsorption process is mostly dependent on ionic interactions. Consequently, the application of endotoxin-specific sorbents is restricted of those contaminated products which are formulated at low ionic strength. Under no circumstances should proteins dissolved at elevated ionic strengths be applied directly.

The negatively charged BSA was best decontaminated using poly-L-lysine-Sepharose. Other ligands, such as histamine or polymyxin B, which displayed good clearance rates with *E. coli* culture filtrate (Table 2), where not effective owing to competing interactions with the protein. A low recovery of BSA was therefore observed and the same can also be expected with other negatively charged proteins.

The parameter of most influence, apart from the ionic strength, on both clearance rate and recovery is the pH of the solution applied. Much better results for decontamination of the BSA solution were obtained after decreasing the pH below the isoelectric point of BSA. At alkaline pH the clearance rates dropped drastically in general, which is in agreement with published results [11].

It has been claimed that a very high ionic strength (>3 *M* NaCl) also leads to adsorption of endotoxins [11,13]; however, such high ionic strengths are not realistic. An additional step would be required to remove the salt. At low ionic strength, hydrophobic moieties of the sorbents seemed not to be of great benefit. Hence none of these sorbents behaves like a true "affinity sorbent" for endotoxins.

In general, protein solutions with extremely high endotoxin contamination should not be applied directly to these sorbents. With the flow-rate and column lengths employed in this study, high endotoxin concentrations were always measured in the column effluent. Considering the adsorption isotherms and the applied endotoxin concentration (>7000 EU/ml), one is operating close to or above the plateau region of the isotherm; hence column length, flow-rate and adsorption kinetics are very important parameters during endotoxin adsorption. Maybe the high flow-rate employed in all experiments (2 cm/min) led to partial breakthrough of endotoxins, which might have been adsorbed at lower flow-rates. On the other hand, such high flow-rates are routinely applied to minimize the process time.

5. Conclusions

An endotoxin-specific sorbent for general decontamination of protein solutions seems not to be available. Owing to competition of proteins with endotoxins for adsorption sites, each decontamination process must be optimized separately. DEAE-Sepharose was the most suitable sorbent for the decontamination of positively charged proteins, such as lysozyme, owing to its low cost and high capacity. Other sorbents with DEAE functionalities are probably of similar effectiveness.

Clearance of negatively charged proteins is problematic and typically does not lead to high clearance rates. Of the five sorbents investigated, poly-L-lysine-Sepharose was most appropriate for the decontamination of BSA solutions; however, the results were only satisfactory at the lowest endotoxin level employed (33.7 mg/ml).

At very large endotoxin contaminations, direct application of protein solutions to these sorbents is not effective. In such extreme cases ultrafiltration should be used as an initial step in order to reduce the concentration, especially of large agglomerates of endotoxins, whereupon the endotoxin-specific sorbents can be employed in a subsequent step.

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