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Capture of bacterial endotoxins using a supermacroporous monolithic matrix with immobilized polyethyleneimine, lysozyme or polymyxin B

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Abstract

Bacterial endotoxins (BEs) are integrated part of *Escherichia coli*, a microorganism widely used for the production of recombinant proteins. BEs should be eliminated in the course of down stream processing of target protein produced by these bacteria. Supermacroporous monolith (continuous bed) columns, so called cryogel columns, with immobilized polyethyleneimine (PEI), polymyxin B (PMB) and lysozyme were employed for BEs capture. Due to the large interconnected pores it was possible to use cryogel columns at flow rates as high as 10 ml/min. The columns packed with Sepharose CL-4B with immobilized PEI, PMB and lysozyme were impossible to use at these high flow rates due to the collapse of the bed. The dynamic capacities of the cryogel columns were nearly independent of the flow rate. In the presence of EDTA, BEs were quantitatively captured from mixtures with a model protein, bovine serum albumin (BSA) at pH 7.2 with practically no protein losses. At pH 3.6 BEs were captured directly from non-clarified *E. coli* cell lysate resulting in more than 10^4 times BEs clearance.

Keywords: Supermacroporous gel; Monolith column; Endotoxin; Affinity chromatography

1. Introduction

Bacterial Endotoxins (BEs), also known as lipopolysaccharides (LPS), are an integrated part of outer membrane of Gram negative bacteria. BEs are composed of three parts. The first part is a lipid part called lipid A, which has two 1,6-linked phosphoryl glucosamines acylated asymmetrically with four hydroxylated fatty acids (Seydel et al., 2003). The second part is a core part consisting of an inner core region with three 2-keto-3-deoxyoctonic acid residues linked to heptose residues and an outer core hexose

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region (Petsch and Anspach, 2000). The third part is *O*-specific part consisting of repeating (4–40) oligosaccharides units with 3–8 monosaccharides in each unit (Petsch and Anspach, 2000). Lipid A is the most conserved part of BEs and it is responsible for the toxicity, however the *O*-specific part is responsible for the antigenicity of BEs. BEs are heterogeneous both in size and composition and are amphiphilic in nature having both a hydrophilic part (the phosphoric acid groups and sugar part) and a hydrophobic part (the fatty acids part in lipid A) (Hirayama and Sakata, 2002). Due to their amphiphilic character, BEs are prone to form micelle-like structures in aqueous media with molecular weights up to 1000 kDa (Santos et al., 2003).

Upon administration in blood stream, BEs cause inflammation and septic chock (Liu et al., 1997). The general threshold level of endotoxin set by pharmacopoeias world-wide for intravenous applications is 5 endotoxin units (EU) per kg body weight per an hour with 1 EU being equal to 100 pg BEs (Hirayama and Sakata, 2002). The amount of BEs allowed in pharmacological protein preparations depends on the intended use. For the proteins that are administrated in relatively low doses, the regulatory demands are modest, for example insulin and α-interferon could contain up to 10 and 100 EU/mg, respectively (Petsch and Anspach, 2000). However, for the preparations of antibodies and human serum albumin that are administered in large amounts, the allowed amount of BEs per mg of protein is extremely low.

As BEs are integral part of *Escherichia coli*, a microorganism widely used for the production of recombinant proteins, there is always a risk of BEs co-purification with the target product. BEs concentrations in starting material varies from less than 100 EU/ml in cell culture supernatants to more than 2,000,000 EU/ml in supernatants after homogenization of high cell density bacterial cultivations (Petsch and Anspach, 2000). Various methods have been used for BE removal from non-protein preparations including ion-exchange chromatography, ultra-filtration and sucrose gradient centrifugation (Hirayama and Sakata, 2002). Affinity adsorption methods have been proven to be efficient for BE removal from protein solutions (Anspach, 2001). Selective endotoxin adsorption implies the use of negative chromatography. In this technique, the target product (protein of interest) passes through the column, whereas the contaminants are bound. LPS-binding proteins like endotoxin-binding protein, bactericidal/permeability-increasing protein and palate lung nasal epithelial clone protein have a high specificity towards BEs (Ghafouri et al., 2004). Alternatively, less specific, but more cheap and robust ligands like polyethyleneimine (PEI), lysozyme, Polymyxin B (PMB), polyhistidine, polyhistamine, polylysine and histidine have been used for BEs removal (Anspach, 2001; Matsumae et al., 1990).

Traditionally these ligands were immobilized on matrices like Sepharose used for protein chromatography. These matrices have some inherent disadvantages when being used for negative chromatography: the columns have high flow resistance resulting in pronounced pressure drops when pumping feed through the column; slow mass transfer especially of large entities like BEs micelles due to the diffusion in and out of the pores; impossibility to process turbid feeds due to the column blocking by particulate material.

The development of supermacroporous monolith (continuous bed) columns has opened new perspectives for selective capture of biomolecules at high flow rates from non-pretreated feeds like cell homogenates (Arvidsson et al., 2003) or cell suspensions (Dainiak et al., 2004). Monoliths have many advantages over beds of packed beads, as they are inexpensive, the production can be performed in situ in different formats from 96 well plate format (Hanora et al., in press) to large column for industrial application with the bed volume up to 8000 ml (Podgornik et al., 2004). The mass transfer in monoliths is due to convection rather than diffusion as in case of packed bed columns allowing for operation at high flow rates without loosing resolution (Bedair and Rassi, 2004) or capacity (Podgornik et al., 2004). Indeed, fast processing is highly advantageous for decontamination using negative chromatography. In this study, supermacroporous monolith columns with immobilized ligands, polyethyleneimine, lysozyme, and polymyxin B were evaluated for BEs removal from model BEs–protein mixtures and crude cell lysate.

2. Material and methods

2.1. Chemicals and reagents

Polymyxin B (MW 1450 Da), polyethyleneimine (MW 60,000 Da), lysozyme (MW 14,300 Da), pic-

rylsulfonic acid (2,4,6-trinitrobenzene sulfonic acid hydrate; TNBS) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA). Sepharose CL-4B was obtained from Amersham Biosciences (Sweden). All other chemicals were of analytical grade and commercially available. The supermacroporous monolith columns with bed volume of 2 ml (2.5 cm × 1 cm i.d.) produced from polyacrylamide and carrying reactive epoxy groups were provided by Protista Biotechnology AB (Lund, Sweden) in dry state.

2.2. BEs preparation

BEs were prepared from *E. coli* cells according to Westphal and Jann (1965). Briefly, cells were grown in Luria Bertani media (LB) until the OD_{600} reached 0.6. The cells were centrifuged and the cell pellet was suspended in water. The cell suspension was treated with preheated 90% phenol solution. The mixture was vigorously shaken at a temperature of 65–70 °C. The emulsion was then centrifuged for 30 min at 1000 × *g* and the aqueous layer containing endotoxin was separated. The aqueous extract was dialyzed and lyophilized. The lyophilized preparation contained about 50–60% bacterial RNA. To purify BEs further, the lyophilized preparation was dissolved in 5 ml 0.5 M NaCl. A 2% solution of hexadecyl-trimethylammonium bromide (CTAB) in 0.5 M NaCl was added until the amounts in weight between the CTAB and crude extract was about 1.5:1 and the precipitate was removed by centrifugation for 10 min at 11000 × *g*. The supernatant was poured into a 10-fold volume of ethanol. The precipitate formed was removed by centrifugation, dissolved in water, dialyzed against deioinized water, and lyophilized.

2.3. BEs assay

The endotoxin content was assayed using a quantitative, chromogenic LAL assay (endpoint method) following the instructions of the supplier. The released amount of *p*-nitroaniline was measured at $\lambda_{max} = 405$ nm and endotoxin from *E. coli* O111:B4 was used as standard. Endotoxin (LPS) was hydrolyzed by acetic acid hydrolysis. A 200 μl of purified extract was added to 800 μl of 1% SDS dissolved in 20 mM sodium acetate buffer pH 4.5 and the mixture was heated at 100 °C for 2 h. The mixture was lyophilized. The lyophilized substance was dissolved in 100 μl water. To remove SDS, 200 μl acidified ethanol (20 ml ethanol acidified with 100 μl of 4 M HCl) was added and centrifuged. The pellet was washed three times with 200 μl ethanol and was extracted with chloroform:methanol:water (12:6:1) mixture. The mass spectrometrical analysis of the endotoxin preparation was performed using a QSTAR® hybrid pulsar-*i* instrument (Applied Biosystems, Foster City, CA) equipped with a nano spray ion source. The endotoxin containing sample (10 μl) was desalted using a C_{18} zip tip (Millipore, Billerica USA) and was applied to a metal-coated fused silica capillary needle (Proxeon, Denmark). Data collection was performed with connected software (Analyte QS) from Applied Biosystems. Tandem mass spectrometry was performed on selected parent ions.

2.4. Ligand immobilization

Sepharose 4B was activated with bisoxirane according to Hermanson et al. (1992) for the introduction of reactive epoxy groups. Briefly, 100 ml of Sepharose CL-4B was washed with water, suspended in 75 ml of 0.6 M NaOH containing 150 mg sodium borohydride with stirring. Seventy-five milliliters of 1,4-butandiol diglycidyl ether was added slowly with continuous stirring and the mixture was stirred overnight at room temperature. Finally, the reaction mixture was washed with water to remove non-reacted reagents. Cryogels were wetted with five column volumes (CVs) of deionized water and sodium phosphate buffer pH 7, respectively. Sodium phosphate buffer pH 7.2 containing either 10 mg/ml of PEI or lysozyme or 2 mg/ml polymyxin B was used for coupling ligands to epoxy-activated monolith columns and activated Sepharose 4B as described by Kumar et al. (2003).

2.5. Chromatographic experiments

The chromatographic experiments were performed using a Biologic DuoFlow Chromatographic System (Bio-Rad, Hercules, CA, USA). The supermacroporous monoliths with immobilized ligands were put into FPLC columns (Pharmacia, Uppsala, Sweden) with 10 mm inner diameter. Alternatively the column (2.5 cm × 1 cm i.d.) was packed with Sepharose CL-4B

with immobilized ligands. The columns were equilibrated with 10 column volumes using various buffers ranging from pH 2 to 11 (100 mM sodium phosphate, 100 mM glycine and 100 mM sodium acetate buffers). The samples were dissolved in the corresponding buffer and loaded to the column at various flow rates 0.1, 1 and 10 ml/min. Washing was performed with at least 10 CVs of the corresponding equilibrating buffer. Elution was carried out with 2 M NaCl at a flow rate of 1 ml/min. The absorbance was monitored at 280 nm and the fractions corresponding to the eluted peaks were collected.

2.6. BEs capture from BEs—bovine serum albumin solutions

A solution of BE and bovine serum albumin was prepared in 0.1 M sodium phosphate buffer with and without 0.01 M EDTA at different pH values. The BEs concentration was adjusted to be equal to the dynamic capacities at 5% breakthrough of the corresponding columns. The solution was applied to monolith columns with immobilized ligands as mentioned above at a flow rate of 1 ml/min. Protein concentration was determined using BCA protein assay reagent according to the established method (Smith et al., 1985). BEs concentrations were assayed by measuring the absorbance at 280 nm taking into account the contribution of BSA into absorbance at 280 nm, which was calculated from the results of BCA protein assay.

2.7. BEs capture from E. coli cell lysate

E. coli cells were cultivated as described above until the OD_{600} reached 4. Twenty-five milliliters portions of cultural liquid were centrifuged and the pellets were suspended each in 1 ml of the running buffer (0.1 M sodium phosphate pH 7.2 alternatively 0.1 M glycine buffer pH 3.6). The suspensions were sonicated and the cell homogenates were applied directly to monolith columns with immobilized ligands at a flow rate of 1 ml/min. The columns were washed with the 10 CVs of the corresponding running buffer. Elution was performed with 2 M NaCl in the corresponding running buffer. Absorbance at 280 nm was measured and BEs were assayed using LAL test.

2.8. Cleaning in place and ligand leakage

Ten column volumes of 0.5 M NaOH were applied to supermacroporous monolith with immobilized PEI at flow rate of 4 ml/min, followed by deionized water. Sodium hydroxide eluate was collected and neutralized. Part of the neutralized eluate was dialyzed, freeze dried, resuspended in 1 ml of 0.1 M sodium carbonate buffer pH 9 and finally filtered using a filter disk with pores of 0.2 μm. Half milliliter of neutralized or concentrated eluate was added to 0.5 ml of 170 μM TNBS dissolved in 0.1 M sodium carbonate buffer pH 9, incubated for 5 min at room temperature. The presence of PEI was determined by measuring absorbance at 350 nm.

3. Results and discussion

3.1. BEs preparation and characterization

BE has been purified from *E. coli* cells and LAL test confirmed the endotoxin activity in purified preparation to be 5×10^7 endotoxin units/ml. The structure of purified endotoxin was confirmed by mass spectrometry. The mass spectrum had a signal peak at *m/z* 1797 (Fig. 1 and structure I Fig. 2B), which was corresponding to lipid A moiety (Karibian et al., 1999). The negative-ion mode of mass spectrometry promoted the fragmentational cleavage between and within the glucosamine residues resulting in the signal peak at *m/z* 710 (structure IV Fig. 2B) which represented the glucosamine I moiety (reducing) (Karibian et al., 1999). Signal peak at *m/z* 1244 (structure II Fig. 2B) corresponded to lipid A with the following modifications: the glucosamine I (reducing) loses phosphate group at position 1 and the double bond is formed between C1 and C2, glucosamine II (distal) loses the primary fatty acid 3-hydroxy fatty acid (3-OH C_{14}) in position 3′ and the double bond is formed between C3′ and C4′. The signal peak at *m/z* 1226 (structure III Fig. 2B) corresponded to the following: the loss of primary 3-hydroxy fatty acid at position 3 and phosphate group at position 1 in glucosamine I (reducing), the loss of the secondary fatty acid (C_{14}) attached to primary fatty acid in position 3′ of glucosamine II (distal) and the formation of double bonds between C1–C2, C3–C4 and C3–C4 in primary fatty acid at position C3′ (Lee et al., 2004) (Fig. 2).

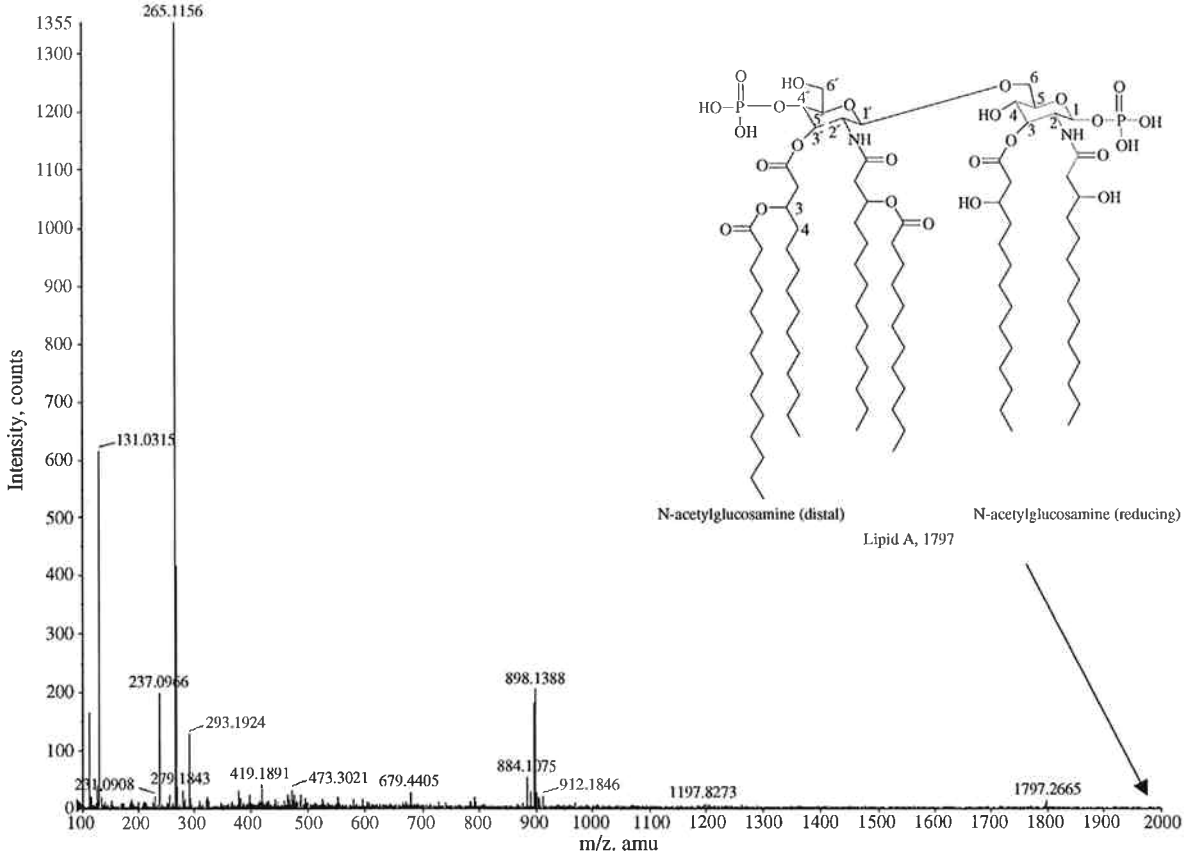

Fig. 1. Mass spectrum of purified endotoxin showing lipid A peak at *m/z* 1797 and the structure of lipid A.

3.2. Chromatographic behavior of BE on supermacroporous monolith columns with different immobilized ligands

The chromatographic behavior of purified BE has been studied on affinity columns produced by immobilization of polymyxin B, lysozyme or polyethyleneimine to supermacroporous polyacrylamide monoliths, so called cryogels. Cryogels (from the Greek κριοσ (kryos) meaning frost or ice) are produced by polymerization of monomers and cross-linker at subzero temperatures when most of the solvent, water, is frozen while the dissolved substances (monomers or polymers) are concentrated in small non-frozen regions. The reaction proceeds in these non-frozen regions while the crystals of frozen solvent perform like porogen. After melting the ice crystals, a system of large interconnected pores is formed. The size and shape of the pore formed depends on the initial concentration of reagents in solution and the freezing conditions. The cryogel have large interconnected pores of 5–100 μm (supermacroporous), non-porous and dense walls that were clearly shown by SEM picture (Fig. 3). The large interconnected pores endow the monoliths cryogels with unique elastic and spongy morphology that make these monoliths fundamentally different from the well known rigid poly (glycidyl methacrylate) monoliths (Svec and Frechet, 1999; Peters et al., 1997, 1999) or superporous molded monoliths agarose (Gustavsson and Larsson, 1999; Gustavsson et al., 1998). The most part of water (70%) is squeezed mechanically from the pAAm monoliths. The functional epoxy groups on the surface of the monoliths allowed their modification with required ligands at high pH. The cryogel allows direct processing of cell suspensions with no need in pretreatment with

flow rates up to 2000 cm/h at normal pressure chromatography mode (Arvidsson et al., 2003; Dainiak et al., 2004; Plieva et al., 2004a,b). Thus supermacroporous monoliths are promising as matrices for negative chromatography aimed at capturing BEs.

Three ligands selected were polymyxin B, a cyclic cationic decapeptide antibiotic with pK >9 for the α-amino group and pK = 10 for δ-amino group of diaminobutyric acid, respectively (Petsch et al., 1997); PEI is a highly branched polymer (the ratio of primary, secondary and tertiary amines is 1:2:1) of molecular weight 60 kDa with pK >9 for primary amino groups and pK >10.5 for secondary amines (Petsch et al., 1997, 1998b); and lysozyme from hen egg white is a small and relatively stable protein with MW 14.4 KDa and pI of 11.2. Lysozyme is capable of binding oligosaccharides and hydrolyzing preferentially the β-1,4 glucosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucoseamine which occur in the mucopeptide cell wall structure of certain microorganisms. There are 39 amino groups (with equal or more than 30% accessibility) at the protein surface available for binding negative moieties. Thus, ligands are positively charged under neutral conditions. However, BEs are negatively charged under neutral conditions due to phosphate groups with pK_1 1.3 and pK_2 8.2 (Hou and Zanieweki, 1990; Petsch et al., 1998a). Apart of electrostatic interactions, one could expect some BEs binding to PEI or polymyxin B via hydrophobic interactions (Hirayama and Sakata, 2002) and some BEs binding to lysozyme due to the protein affinity towards oligosaccharides.

All three ligands bind BEs efficiently at pH 7.2, the bound BEs were quantitatively eluted with 2 M NaCl (Fig. 4). As the columns were saturated with BEs (BEs were applied until nearly the complete breakthrough) the eluted peaks represented the static binding capacity,

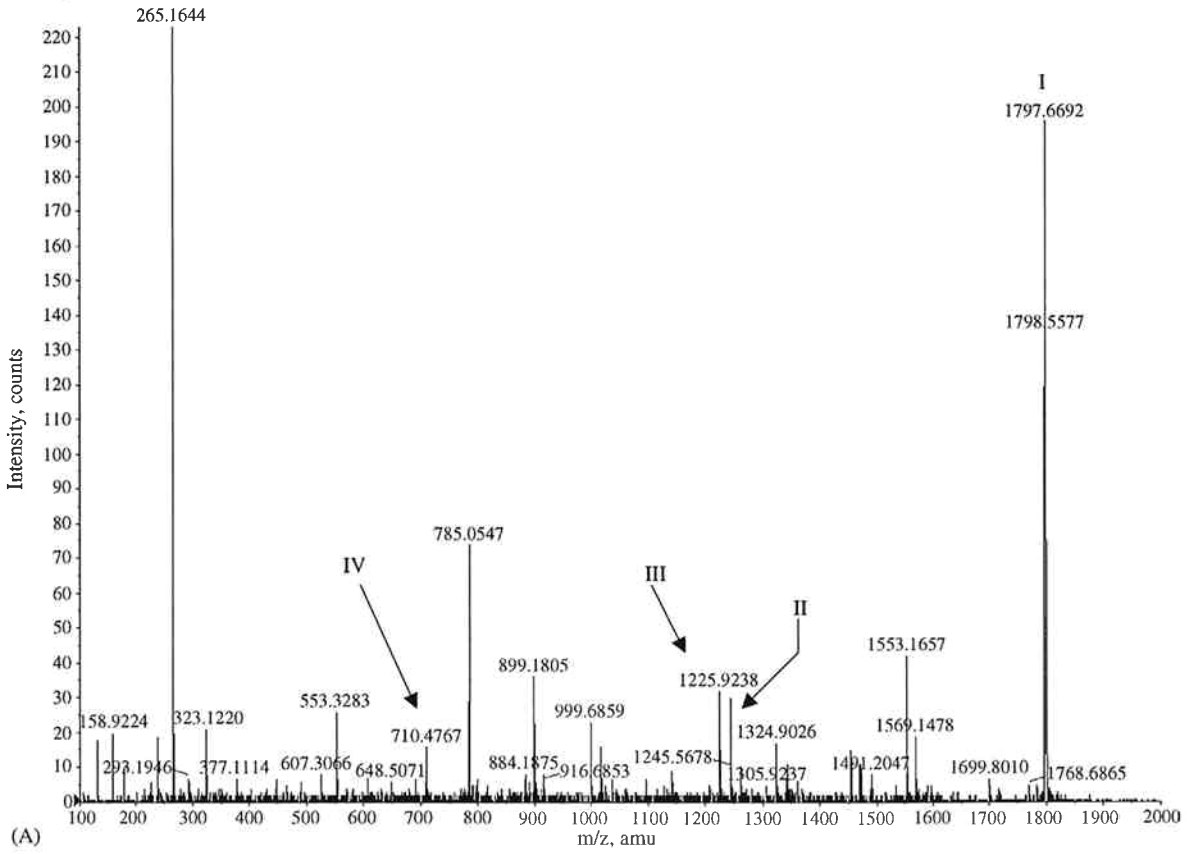

Fig. 2. (A) Negative-ion mode nanospray mass spectrum of lipid A; (B) the fragmentational structures corresponding to the peaks.

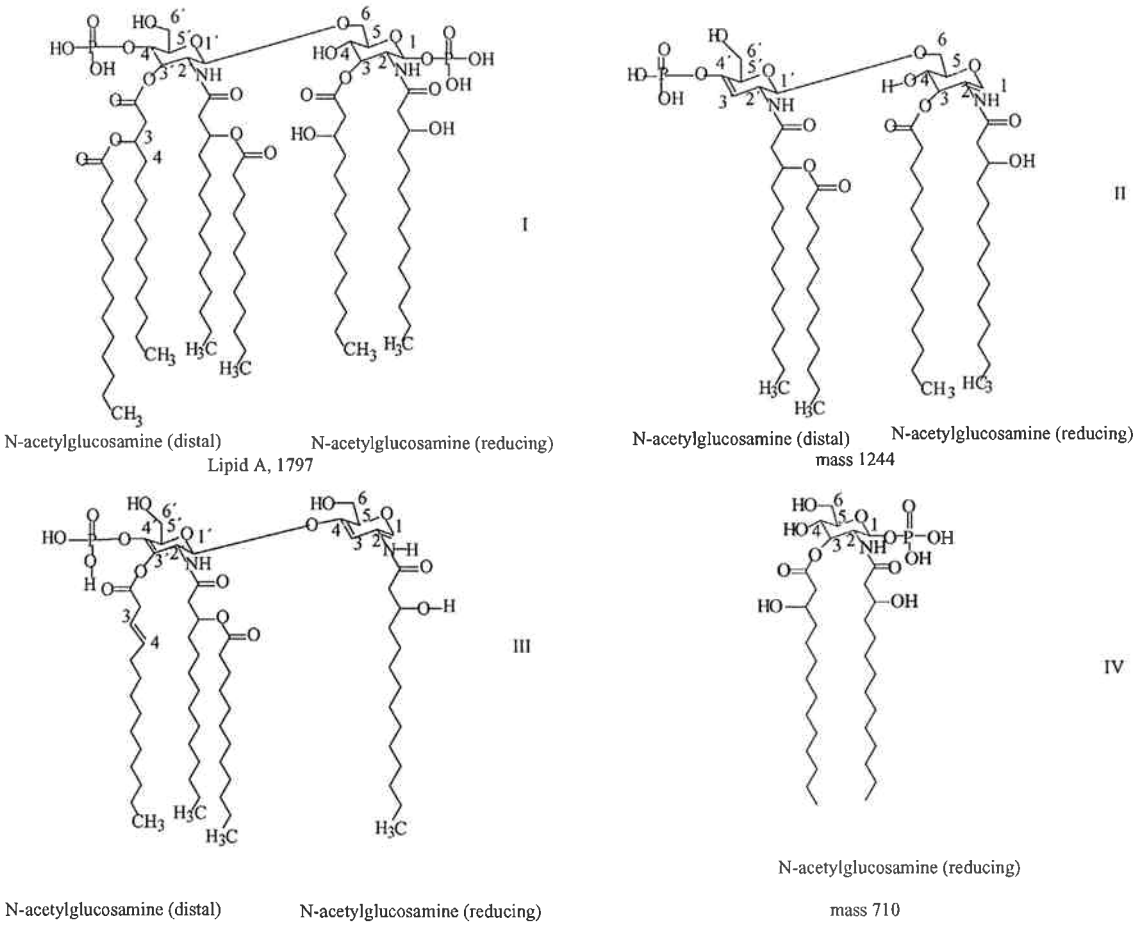

Fig. 2. (*Continued*).

which was much higher in the case of PEI as compared to the other two ligands.

There was no BEs binding to PEI-ligands or lysozyme-ligands at high pH values probably due to the deprotonation of ligands and their loss of positive charge. Contrary, below pH 7, the efficiency of BEs capture improved with decreasing pH as judged by decreasing area of small breakthrough peaks (Fig. 5A and B).

The BEs aggregation in aqueous media was presumed to be the reason for the observed breakthrough peaks. The large micelle-like structures were formed by BEs due to non-polar interactions between neighboring alkyl chains as well as due to the bridging between phosphate groups by bivalent cations like Ca(II) (Petsch and Anspach, 2000; Santos et al., 2003). The micelle could pass non-hindered through the pores of superporous monolith columns without being bound by the ligands. To investigate the effect of micelle formation on chromatographic profile, the eluted fractions from the column with immobilized lysozyme were combined, dialyzed, lyophilized, dissolved in sodium phosphate buffer pH 7.2 and re-loaded on the column. Despite that all the material applied on the column has been already once bound to the column, the breakthrough peak was still observed, indicating that whatever was in the peak was aggregated in situ. Mass spectrometry confirmed the presence of BEs in all three fractions—eluted after the first chromatographic run, breakthrough and eluted fractions after

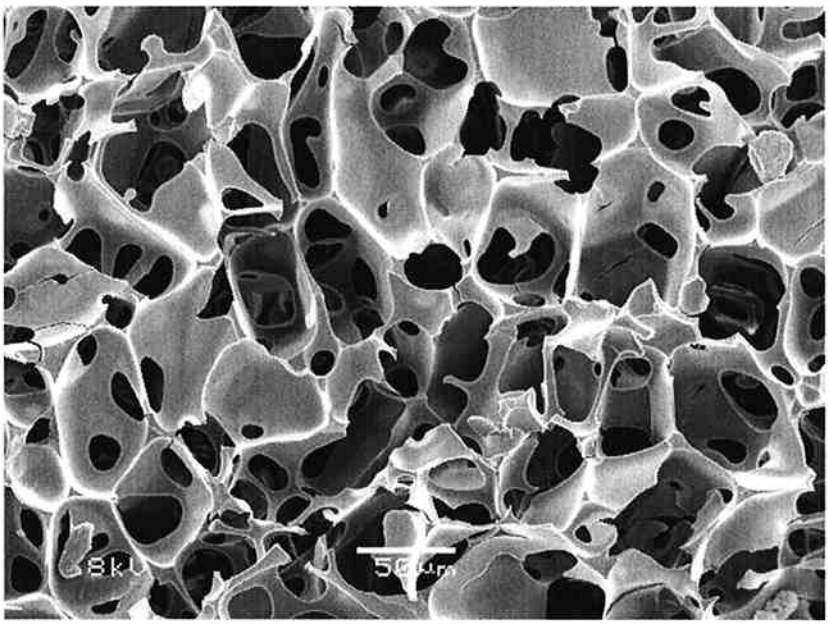

Fig. 3. Scanning electron microphotograph of the radial cross-sections of cryogel. The sample was fixed in 2.5% glutaraldehyde in 0.12 M sodium phosphate buffer, pH 7.2 overnight, post-fixed in 1% osmium tetroxide for 1 h, dehydrated in ethanol and critical point dried. The dried sample was coated with gold/palladium (40/60) and examined using a JEOLJSM-5600LV scanning electron microscope.

re-chromatography (data not shown). This observation agreed well with the data on the formation of BEs aggregates with a size of 119 nm at concentrations below 14 μg/ml and larger micelle-like aggregates of 190 nm in size at concentrations above 40 μg/ml (Santos et al., 2003). The dissociation constant for BEs aggregation was estimated to be 34 nM at pH 7.4 and was relatively independent of temperature and ionic strength (Takayama, 1996). The dissociation rate of micelles is very low (Petsch and Anspach, 2000).

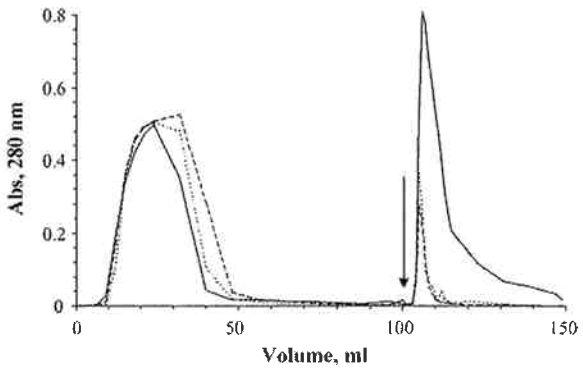

Fig. 4. Chromatographic profiles of endotoxin breakthrough and elution on superporous monolith columns with immobilized PEI (solid line), lysozyme (dash line) and polymyxin B (dotted line); arrow indicates the start of elution. Experimental conditions: 0.1 M sodium phosphate buffer pH 7.2 was used as running buffer and elution was carried out with 2 M NaCl in the running buffer at a flow rate of 1 ml/min.

The BEs micelles even at low BEs concentrations have higher stability than micelles formed by simple detergents. The addition of a surfactant, Tween 80 in concentrations 0.5 or 5 of its critical micelle concentration (CMC) had no effect on the breakthrough portion of BEs when the sample was applied to a lysozyme–ligand column (data not shown).

Thus, acidic conditions happened to be the most appropriate for BEs capture probably due to the decreased tendency of micelle formation. However, the feasibility of using this technique for decontamination of target protein from BEs depends strongly on the stability of the target proteins. Two pH values were selected for further studies, pH 7.2 as the medium which is appropriate for the decontamination of target protein as most of the recombinant proteins are stable under neutral conditions, and pH 3.6 as the medium which is appropriate for the decontamination

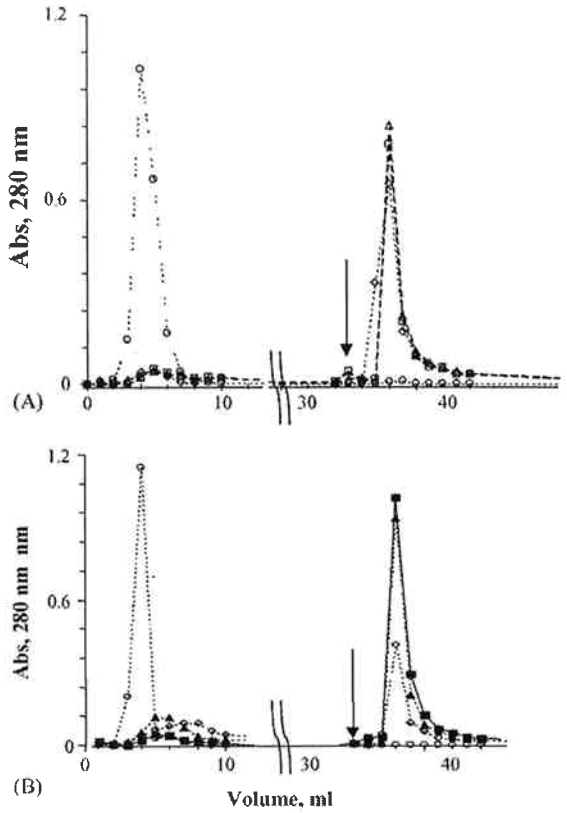

Fig. 5. Chromatographic profiles of endotoxin breakthrough and elution on superporous monolith columns with immobilized PEI (A) and lysozyme (B) at different pH values. Experimental conditions: BEs were applied in 1 ml of the running buffer followed by washing and elution with 2 M NaCl in the running buffer at a flow rate of 1 ml/min. Sodium phosphate buffers are as follows: pH 11 (open circle with dotted line), pH 7.2 (open rhomb with dotted line), pH 4.6 (open square with dotted line), and pH 3.8 (open triangle with dashed line). Glycine buffers are as follows: pH 3 (closed square with straight line) and pH 2 (closed triangle with dotted line).

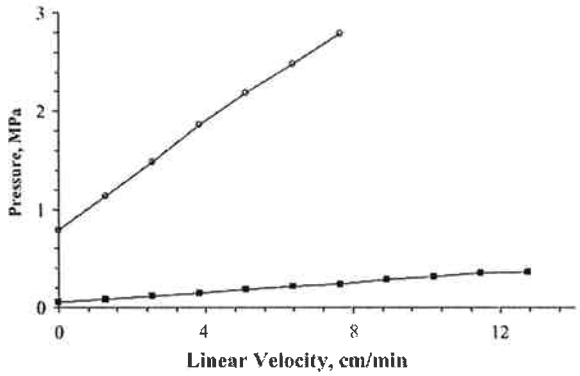

Fig. 6. The pressure drop for superporous monolith column (closed square) and a column of similar size (2.5 cm × 1 cm i.d.) packed with Sepharose CL-4B (open circle). The experiments were carried out on Bio-Rad FPLC system, where the flow rate was increased stepwise every one minute. The backpressure was recorded and the experiment was stopped when the Sepharose CL-4B bed was compressed.

of wastewater, where the stability of other components is irrelevant.

The most important characteristic of the chromatographic decontamination process is the dynamic capacity of the column which indicates how fast and how efficient BEs could be captured. The dynamic capacities at 5% BEs breakthrough level of the columns with different ligands are presented at two flow rates 1 and 10 ml/min, respectively (Table 1). For comparison, the capacities for the column packed with Sepharose CL-4B with the same ligands immobilized are presented at flow rate of 1 ml/min. It was impossible to use Sepharose column at flow rates above 10 ml/min due to the progressively increased pressure drop and finally collapse of the bed (Fig. 6).

At low flow rate, the dynamic capacity of the column with immobilized polymyxin B was higher at pH 7.2

Table 1
Relative dynamic capacities at 5% BEs breakthrough for cryogel and Sepharose columns with immobilized polyethyleneimine, lysozyme and polymyxin B at different pH values and different flow rates

Ligands	Polyethyleneimine			Polymyxin B			Lysozyme		
Matrix	Cryogel		Sepharose	Cryogel		Sepharose	Cryogel		Sepharose
pH	3.6	7.2	7.2	3.6	7.2	7.2	3.6	7.2	7.2
Flow rate ml/min									
1	5.5	1.7	6	8.4	3.5	12	2.3	2	6
10	5.5	3.3	–	5.8	2.9	–	3.5	3	–

Relative dynamic capacities are presented as absorbance units of BE at 280 nm per 1 ml of column volume.

than that of the columns with immobilized PEI and lysozyme, respectively, probably due to some specific interactions of polymyxin B with the lipid A part of BEs on top of ion-exchange interactions characteristic for all three ligands (Petsch et al., 1998c). However at pH 3.6, the differences in capacities were less pronounced.

At low flow rate, the dynamic capacities of Sepharose-based absorbents were higher than that of supermacroporous monolith columns for all three ligands. It is not surprising since Sepharose CL-4B has much smaller pores than supermacroporous monoliths and hence larger surface available for ligand immobilization. On the other side, smaller pores in Sepharose CL-4B entail higher pressure drops in Sepharose-packed columns as compared to supermacroporous monolith columns and impede using Sepharose-packed columns at flow rate as high as 10 ml/min.

However, the supermacroporous columns perform at high flow rate nearly as well as at low flow rate for polymyxin B-ligand. It is interesting to note, that for PEI- and lysozyme-ligands, the capacity even increased with the flow rate. A similar behavior has been observed earlier for supermacroporous monoliths with ion-exchange or iminodiacetate ligands (Plieva et al., 2004b). The rationale behind could be as follows. As the supermacroporous monoliths have very large pores and present a very low flow resistance, at low flow rates the liquid could pass through the column using only some of the pores, probably the largest ones, so some of the smaller pores are not exposed to the mobile phase resulting in a decrease in the apparent capacity. With increasing flow rate, the mobile phase starts passing through smaller pores as well as through the larger ones. Hence, more ligands are exposed to the mobile phase and more BEs could be bound, the capacity increases. The even further decrease in capacity for supermacroporous monolith column with immobilized PEI from 1.7 to 0.5 absorbance units, respectively, when the flow rate was decreased to an extremely small value of 0.1 ml/min indicates in favor of this assumption.

3.3. BEs capture from protein solutions

A prerequisite for decontamination of protein solutions from BEs is a fast and selective binding of endotoxin to the adsorbent without affecting the recovery of the protein. BSA, a slightly acidic protein with p*I* of about 4.6 (Chaiyasut and Tsuda, 2001), was used as a model protein for optimizing the conditions for BEs decontamination (Table 2). Lysozyme happened to be the least suitable ligand for BEs capture in the presence of protein at pH 7.2, allowing only for around 60% of BEs to be captured. The incomplete BEs capture could be attributed to BEs binding to BSA with a dissociation constant $K_D \sim 4.3 \times 10^{-6}$ M under neutral conditions (Petsch et al., 1998c). It was reported that, the binding due to the electrostatic interaction between lysozyme and lipid A-phosphate groups with a [LPS]:[lysozyme] molar ratio 3:1 (Brandenburg et al., 1998). However, the association constant for BEs to bind to PEI immobilized on Nylon membrane is at least two order of magnitude higher than that for binding to immobilized lysozyme (Petsch et al., 1998a). The efficient (around 95%) capture of BEs along with quantitative protein recovery in the eluate was achieved either at pH 4.7 and 6 for lysozyme, or pH 4.7 and 7.2 for PEI, respectively. While the dissociation constant of polymyxin B–BEs is 2.46×10^{-9} M (Minobe et al., 1988), quantitative recovery of bound BEs were obtained at pH 6 and 7.2. In these cases probably, the stripping off BEs from the complex with BSA takes

Table 2
Recoveries of BSA and BEs in different fractions after passing through cryogel columns with immobilized polyethyleneimine, polymyxin B and lysozyme at a flow rate of 1 ml/min

pH	Polyethyleneimine (PEI)			Polymyxin B			Lysozyme		
	BSA (breakthrough)	BSA eluted	BEs eluted	BSA (breakthrough)	BSA eluted	BEs eluted	BSA (breakthrough)	BSA eluted	BEs eluted
7.2	100	0	100	100	0	100	100	0	63
7.2 + 0.01 M EDTA	100	0	100	100	0	100	100	5	90
6	100	0	85	~100	8	100	97	0	94
4.7	~100	5	100	92	6	91	~100	14	100

Recoveries are presented in percentage taking the applied amounts of BSA and BEs as 100%, respectively.