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Review

Chromatographic removal of endotoxin from protein solutions by polymer particles

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Abstract

Endotoxins, constituents of cell walls of gram-negative bacteria, are potential contaminants of the protein solutions originating from biological products. Such contaminants have to be removed from solutions used for intravenous administration, because of their potent biological activities causing pyrogenic reactions. Separation methods used for decontamination of water, such as ultrafiltration, have little effect on endotoxin levels in protein solutions. To remove endotoxin from a solution of high-molecular-mass compounds, such as proteins, the adsorption method has proven to be most effective. In this review, we first introduce endotoxin-specific properties in an aqueous solution, and then provide various methods of chromatographic separation of endotoxins from cellular products using polymer adsorbents. We also provide the design of novel endotoxin-specific polymer adsorbents.

Keywords: Reviews; Polymer particles; Proteins; Endotoxin

Contents

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1. Introduction

Endotoxins are an integral part of the outer cellular membrane of gram-negative bacteria and are responsible for organization and stability [1]. In the biotechnology industry, gram-negative bacteria are widely used to produce recombinant DNA products such as peptides and proteins. Thus, these products are always contaminated with endotoxins.

The removal of endotoxins from drugs and fluids before use in injections is critical, because of their potent biological activities causing pyrogenic and shock reactions in mammals [2,3]. Among pyrogenic substances, it is well-known that endotoxins produce the highest fevers in mammals [4]. The threshold level of endotoxin set by all pharmacopoeias for intravenous applications, is set to 5 endotoxin units (EU) per kg body weight an hour [5]. It was found [6] that 1 EU corresponds to 100 pg of the standard endotoxin EC-5 and 120 pg of endotoxin from *Escherichia coli* O111:B4. Endotoxins are very stable molecules, resisting extreme temperatures and pH values in comparison to protein [7]. Although the removal of endotoxins from final bioproducts has always been a challenge, it is extremely difficult when the endotoxins are associated with the protein to be purified [8]. Various procedures of endotoxin removal, such as ion-exchange (membrane [9] and filter [10]), ultrafiltration [11,12], extraction [13,14], and sucrose gradient centrifugation [15], were developed for pharmaproteins. These procedures, however, are unsatisfactory with respect to selectivity, adsorption capacity, and recovery of the protein. The selective removal of endotoxin from protein-free solutions must be clearly distinguished from removal from protein-containing solutions. In protein-free solutions, it is easy to remove endotoxins by ultrafiltration taking advantage of the different sizes of the endotoxin and water, or by non-selective adsorption with a hydrophobic adsorbent [16] and or an anion-exchanger [17].

For the removal of endotoxin from final solutions of bioproducts, selective adsorption has proven to be the most effective technique [18,19]. Therefore, the development of adsorbents capable of retaining high endotoxin selectivity under physiological conditions (ionic strength of $\mu = 0.05–0.2$, neutral pH) is keenly pursued.

Recently numerous aminated polymer adsorbents have been developed for removing LPS from protein solutions. This review intends to elucidate the chromatographic properties of various endotoxin adsorbents and then describe recent findings concerning methods for eliminating endotoxins from protein solutions by the adsorption technique. We first introduce the chemical and physical properties in the aqueous solution.

2. Chemical and physical properties of endotoxins

2.1. Chemical structures

About a century ago Richard Pfeiffer named the toxic principle ''endotoxin'' to distinguish it from the already well-known heat-sensitive proteinaceous exotoxins [20]. Endotoxins are an integral part of the outer cell membrane of gram-negative bacteria [1], and consist of a hydrophilic heteropolysaccharide and a covalently bound lipid component; therefore chemically, endotoxins represent lipopolysaccharides (LPS) (Fig. 1) [21]. The molecule of enterobacterial LPS can be subdivided into three structurally distinct segments: a lipid component, called lipid A, a core oligosaccharide, and a long heteropolysaccharide chain, the O-specific chain, which is generally composed of a sequence of identical oligosaccharides, the repeating unit [22]. A variety of nonenterobacterial wild-type strains of phototrophic and some human pathogenic gram-negative bacteria including *Neisseria*, *Acinetobacter*, *Bordetella*, *Bacteroides* and *Haemophilus* form LPS which consist only of the core and lipid A region, thus lacking the O-specific chain [23]. Although not all bacterial strains express endotoxins having the complete poly-

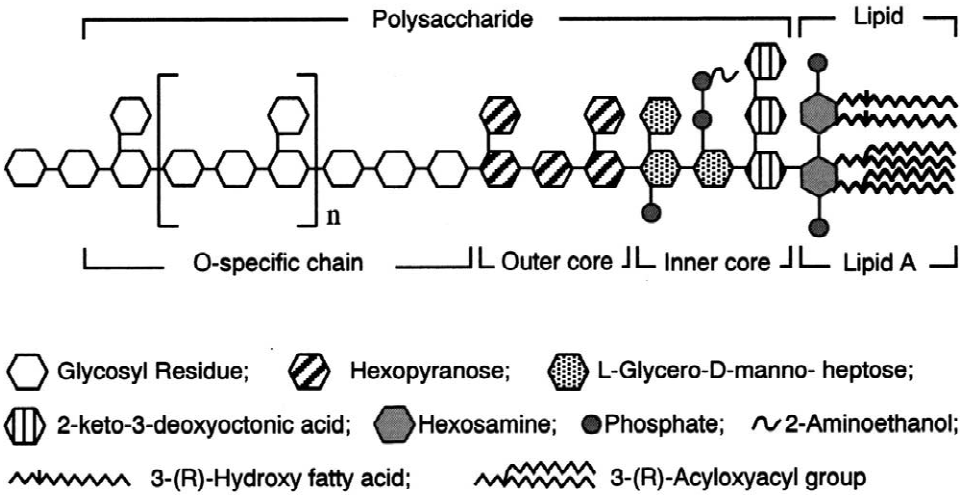

Fig. 1. Architecture and schematic structure of an endotoxin from enterobacteriaceae according to Rietschel et al. [22].

saccharide region shown in Fig. 1, they all contain lipid A. It was also found that the lipid A is a carrier of the endotoxic properties of endotoxin, i.e. the induction of fever and shock [22].

The minimal endotoxin structure required for the viability of the gram-negative consists of lipid A carrying one 2-keto-3-deoxyoctonic acid (Kdo) residue [24]. Fig. 2 shows the chemical structure of the lipid A component in various gram-negative bacteria

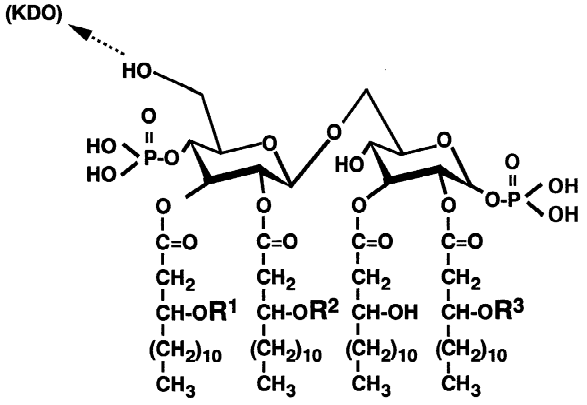

Fig. 2. Chemical structure of various bacterial lipid A. Modified after Seydel et al. [25]. KDO, 2-keto-3-deoxyoctonic acid.

[25]. It was also found that a single chemical structure of the lipid A is hardly to be found even at the same bacterium. Although the structural forms of their lipid A merely differ from each other, the lipid A regions all are partially phosphorylated ($pK_1 = 1.3$, $pK_2 = 8.2$ [26]). Thus endotoxins are amphipathic substances that have both an anionic region (the phosphoric acid groups) and a hydrophobic region (the lipophilic groups).

2.2. Supramolecular structures

The molecular mass of endotoxin monomer is about 1×10^4 as shown in Fig. 1. It is well-known that endotoxins form various supramolecular aggregates in aqueous solutions because of their amphipathic structures (Figs. 1 and 2). Ribi et al. [27] have described that endotoxins were dissociated by the bile salt sodium deoxycholate into nontoxic subunits with molecular masses of about 2×10^4. They also described that when the bile salt was removed by dialysis, the subunits reaggregated in an orderly manner to form a relatively uniform population of biologically active endotoxin particles with an average molecular mass of 5×10^5 to 1×10^6. The aggregate structures have been still further researched by electron microscopy [28,29] and various analytical methods, such as X-ray diffraction, FT-IR spectroscopy and NMR [30]. These results have

shown that in aqueous solutions endotoxins aggregate in lamella, cubic and hexagonal inverted arrangements, such as micelles and vesicles, with diameters up to 0.1 μm. The vesicles are even found in ultrapure water. Monomers are created by using not just the bile salt sodium deoxycholate, but also by detergents (e.g. Triton) and chelators (e.g. EDTA). It is proposed that proteins may also shift equilibria, releasing endotoxin monomers from aggregates [31].

For selective removal of endotoxin from protein solutions, it is necessary to assume endotoxin's form in protein solutions. As shown in Fig. 3, under common protein solution, we assume that endotoxin aggregates form supermolecular assemblies with phosphate groups as the head group, and exhibit a net negative charge because of their phosphate groups that originate from lipid A. These characteristics suggest that ionic interaction plays a part in the binding between the cationic adsorbent and phosphate groups of the endotoxins. When hydrophobic adsorbents are used in protein solutions, it is suggested that there is also this hydrophobic binding between the adsorbent and the lipophilic groups of endotoxins. These binding processes depend on the properties of proteins (net charge, hydrophobicity) and the solution conditions (pH, ionic strength).

3. Removal of endotoxin from protein-containing solutions by adsorbent

3.1. Adsorbents for selective removal of endotoxin

3.1.1. Activated carbon

Already, adsorption techniques using some activated carbons [32] have been employed for endotoxin removal. However, it has been reported [33] that an activated carbon showed adsorbing activities for both endotoxin and protein when it was used as an adsorbent for removing endotoxin from a plasma-containing solution. Thus, a non-selective adsorbent, such as activated carbons, is not suitable for removing endotoxin from a protein-containing solution.

3.1.2. Anion-exchange chromatography

For endotoxin removal from protein-free solutions, anion-exchangers are used, such as anion-exchange polymeric matrix [10], since endotoxins exhibit net-negative charges because of their phosphate groups originating from lipid A. However, the adsorbing capacity of diethylaminoethanol (DEAE)-Sepharose (Fig. 4) is high for both endotoxin and acidic protein such as bovine serum albumin (BSA) [34]. As a result, anion-exchangers, such as DEAE-Sepharose,

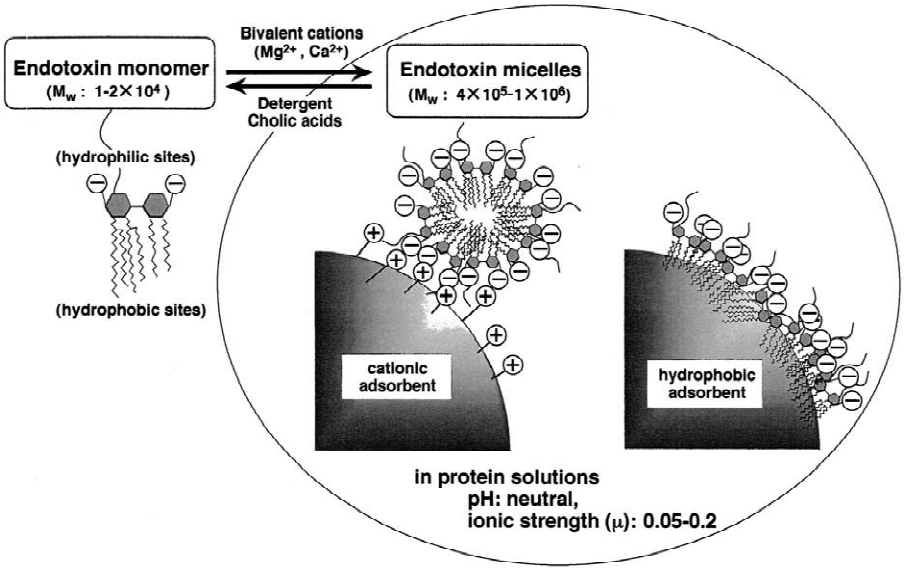

Fig. 3. Schematic diagram of our hypothesis to explain the adsorption behavior of endotoxin aggregates for cationic and hydrophobic adsorbents in protein solutions.

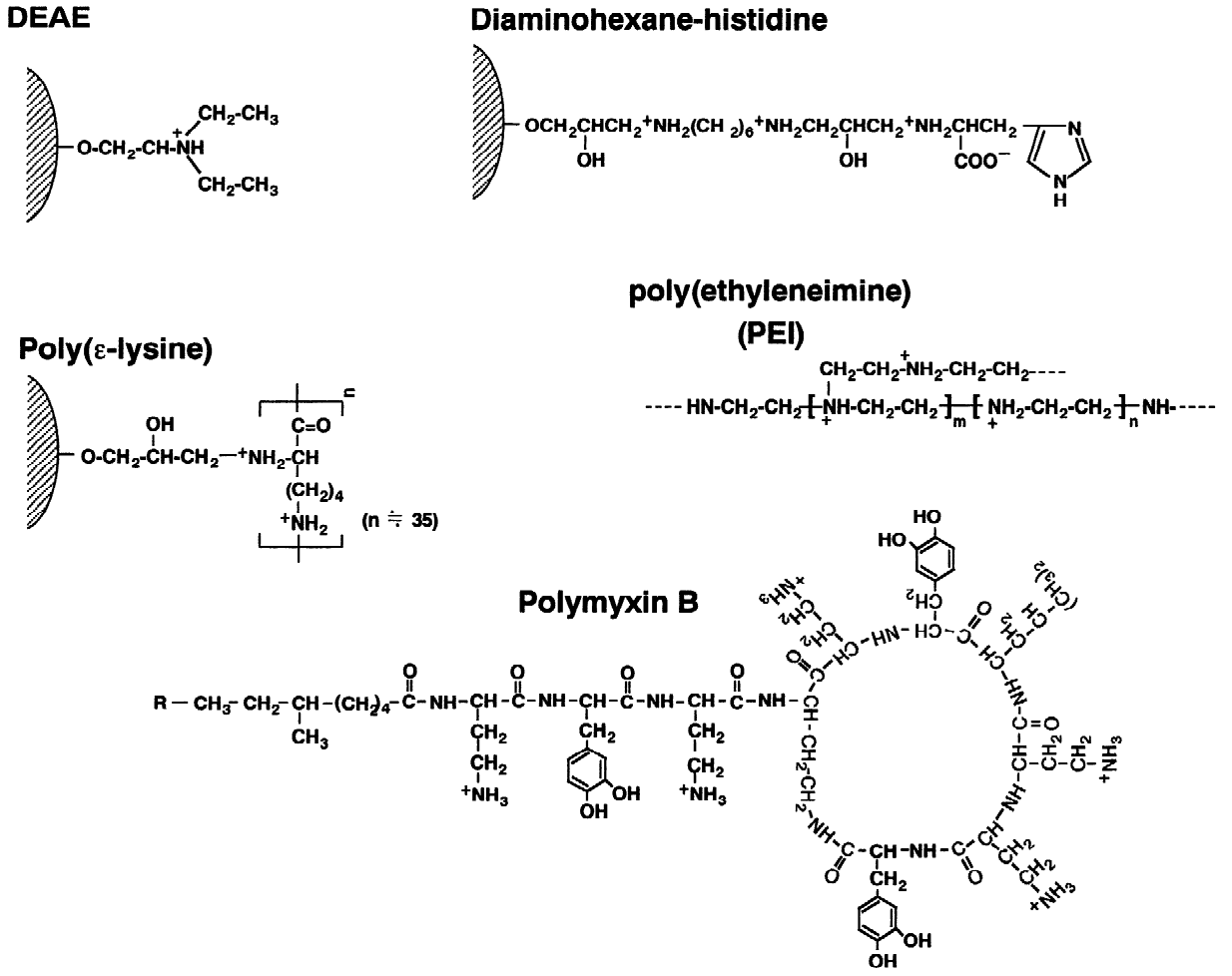

Fig. 4. Structure of endotoxin-selective ligands. Sepharose and cellulose particles are used as the matrix.

are not able to selectively adsorb endotoxin from acidic protein-containing solutions.

3.1.3. Polymyxin B-immobilized Sepharose

It is well-known that cell walls of gram-negative bacteria are deorganized by insertion of the antibiotic polymyxin B [35]. Lopes et al. [36] also reported the surface-active cyclic peptide of polymyxin B (Fig. 4). Polymyxin B is also a basic polymer with positive charges. The ability to break down endotoxin aggregates might be attributed to the existence of a group-selective ligand with the potential to recognize endotoxins of different origins.

Through immobilization of polymyxin B on CNBr-activated Sepharose, Issekutz [37] prepared adsorbent for selective removal of endotoxin. The polymyxin-Sepharose, which is now commercially available, showed endotoxin-removing factors of greater than 10^5 from heavily contaminated culture filtrates (10 μm/ml). Although the polymyxin-Sepharose columns showed high endotoxin-adsorbing activity, protein losses during passage through the column have been noted (loss of bovine catalase, 24% [38]; loss of BSA, 20% [39]). These are due to the ionic interaction between the cationic region of the polymyxin B and the net-negatively charged proteins at low ionic strengths. Furthermore, polymyxin B is not suitable as a ligand for endotoxin

removal from a solution for intravenous injection. Both because it is expensive, and because if any polymyxin were to be released into a solution, it would be physiologically active [40].

3.1.4. Histamine and histidine-immobilized Sepharose

Kanoh et al. [41] discovered that ribonucleic acid has a high affinity for endotoxin and it is very difficult to remove from nucleic acids. Later Minobe et al. [42] considered that if the components of nucleic acid and related compounds, such as adenine, cytosine, histamine and histidine, are immobilized to water-insoluble chromatographic matrices, the matrices obtained could be used as specific adsorbents for endotoxin. Although they reported that an adsorbent having histamine as a ligand showed the highest affinity for endotoxin [43], they later reported that histidine was safer than histamine as the ligand of a specific adsorbent to remove endotoxins from a solution for injection, because of histamine's biological activity [44]. Matsumae et al. [45] described that histidine-Sepharose can be used for the removal of natural endotoxins from various useful cellular products at low ionic strength and around neutral pH. According to Minobe et al., the mechanism of the endotoxin-adsorbing activity is attributable to the simultaneous effects of ionic and hydrophobic interaction, originating from imidazole and the spacer diaminohexane (DAH), respectively (Fig. 4). However, Petsch et al. [46] reported that the endotoxin-adsorbing activity of histidine-Sepharose is mainly caused by properties originating from DAH, but not necessarily from histidine, because of a low pK_a of the imidazole ring (p$K_{imidazole}$ 6.0).

3.1.5. Chromatographic matrices having polycationic ligand

As shown in Figs. 1 and 2, endotoxins are amphipathic substances which have both anionic and hydrophobic regions. According to molecular dynamics, the structure of an endotoxin aggregate is rather flexible compared to that of a protein [47]. It was suggested that endotoxin could also be adsorbed at a high salt concentration (greater than 3 *M* NaCl) using histidine-Sepharose [45]. This is mainly due to the hydrophobic contribution of the ligand. In hydrophobic binding chromatography, similar behavior has been reported using another endotoxin-selective ligand [48].

From these points of view, an endotoxin-selective ligand should have not only cationic properties but also hydrophobic properties. Several cationic polymers with hydrophobic properties have been already used as ligands (Fig. 5). Mitzner et al. [49] reported that poly(ethyleneimine) (PEI) immobilized cellulose beads showed high endotoxin-removing activity in plasma. PEI-immobilized cellulose fibers were also prepared by Morimoto et al. [50] and the PEI-fibers showed greater endotoxin removal from BSA solutions than did the corresponding histidine-Sepharose, and with less dependence on the ionic strength (up to 0.2 *M* NaCl).

In a more recent publication, poly(ε-lysine) was covalently immobilized onto cellulose spherical particles and used for selective adsorption of endotoxin from protein solutions [51]. The poly(ε-lysine)-immobilized cellulose (PL-cellulose) particles can reduce concentrations of endotoxin to 0.1 EU/ml or lower in drugs and fluids for use in intravenous

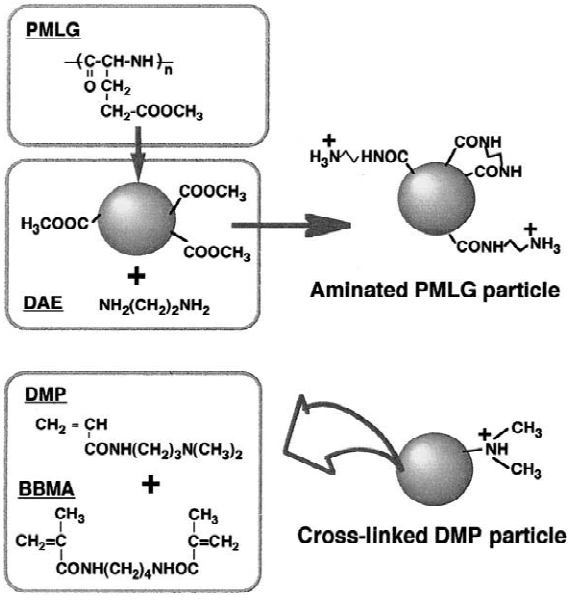

Fig. 5. Structure of endotoxin-selective polymeric matrices with cationic functional groups.

injection, at a neutral pH and at wide ionic strengths of $\mu = 0.05$ to 0.4. These processes did not affect the recovery of even acidic proteins such as BSA. In addition, the PL (degree of polymerization, 35; pK_a 7.6) [52,53] produced by *Streptomyces albulus*, which has become commercially available as a safe food preservative, is more suitable as a ligand than is polymyxin B and histamine.

The high endotoxin selectivity of chromatographic matrices having polycationic ligands, such as PEI or PL, is possibly due to the simultaneous effects of the cationic properties of the ligand and its hydrophobic properties.

3.1.6. Polymeric matrices with cationic functional groups

Immobilization of ligands to microfiltration membranes yielded polymers with cationic functional groups. In Petsch et al., either small ligands, such as histidine, deoxycholate, polymyxin B or DEAE, or polycationic ligands, such as poly(L-lysine) or PEI were immobilized inside hydrophilic polymer networks [46]. They reported that these membranes with ligands deoxycholate, PEI and poly(L-lysine) showed high endotoxin-removing activities. Although these membranes have cationic properties and therefore adsorb net-negative charged proteins, under optimized environmental conditions (ionic strength and pH), their protein recoveries can increase to 88 to 95% [46].

Through immobilization of *N*-actadecylchitosan to carboxylated porous supports, Wakita et al. also prepared cationic lipid membranes [54]. The authors describe that the membranes adsorbed endotoxin selectively from BSA solutions at pHs of 4.3 to 7.0 with ionic strengths of 0.01 to 0.1: endotoxin was removed to levels as low as 1 EU/ml from a BSA solution by a column-wise adsorption.

The selective removal activities of these membranes are due to the simultaneous effects of the cationic properties of ligands and the hydrophobic properties of membranes, and the decreasing effects of the protein-binding capacity when the pH and ionic strengths of the buffer's condition are adjusted.

Furthermore, several polymeric spherical particles with amino groups (Fig. 5) were prepared. Aminated poly(γ-methyl L-glutamate) (PMLG) spheres, having diaminoethane as a ligand, showed a high endotoxin-removing activity from solutions of crude protective antigens, such as *tetanus* toxoids [19] and *pertussis* antigens [55,56]. The aminated PMLG adsorbent showed superior endotoxin binding capacity than commercial endotoxin adsorbents based on histidine and polymyxin even at high ionic strengths, $\mu = 0.2$–0.4 [57]. The spherical PMLG adsorbent can be readily macroreticulated from <500 to 2×10^6 as the pore size (molecular mass exclusion) by adding a diluent, such as di(2-ethylhexyl)phthalate or decahydronaphthalene in the process of sphering [58]. The adsorption of several cellular products, such as BSA and γ-globulin, increased with increasing the adsorbent's pore size [59]. The authors assume that the adsorption of net-negatively charged substances, such as BSA, is caused by their entry into the pores of each adsorbent, but endotoxin aggregates can be adsorbed also at the surface of the adsorbent. This allows for strong endotoxin adsorption and high recoveries of net-negatively charged substances at the same time. This type of adsorbent, however, is unsatisfactory with respect to complete regeneration because it is generally considered that PMLG, having esteratic sites (–CO–O– bonds), is gradually hydrolyzed in an alkaline solution, one of the solvents used for regeneration. The authors later switched to cross-linked *N*,*N*-dimethylaminopropylacryl-amide (DMP) particles, composed of (–CONH–) bonds. The particles were prepared by suspension copolymerization of DMP with *N*-allylacrylamide (AA) or/and *N*,*N*-butylene-bismethacrylamide (BBMA) [60,61]. The adsorbent was able to remove endotoxin from a protein solution, being naturally contaminated with endotoxin, to 1 EU/ml or lower, at $\mu = 0.05$ to 0.2 and at a neutral pH. This process did not affect the recovery of important compounds such as BSA, insulin, myoglobin, γ-globulin and cytochrome *C*. Washing with 0.2 *M* sodium hydroxide followed by 2.0 *M* sodium chloride can completely regenerate the adsorbent.

The selective removal activities of the aminated PMLG and cross-linked DMAPAA particles are due to the simultaneous effects of the cationic properties of amino groups and the hydrophobic properties of the matrix, and the exempting effects on protein molecules when the pore size of the matrix is adjusted.

3.2. Critical evaluation of the analytical and chromatographic results on selective removal of endotoxin with various adsorbents

We consider that the effectiveness of cationic polymer as endotoxin adsorbence increases when a column chromatography process is used. Therefore, by using spherical polymer particles for column packings, this section describes the effect of various factors on the selective removal of endotoxin from protein-containing solution.

3.2.1. Effect of pore size of adsorbent on endotoxin selectivity. In the removal of endotoxins from a dilute endotoxin solution by adsorption, the endotoxin-removing activity depends on the dissociation constant (K_d) between endotoxin and the adsorbent more than on the adsorbing capacity. Table 1 [60] shows the effect of the adsorbent pore size (molecular mass exclusion of polysaccharide, M_{lim}) [63] on the endotoxin-adsorbing capacity and the apparent K_d ($K_{d,app}$) [44]. We previously reported [60,61] that the endotoxin-removing activity for cationic adsorbent increased with increases in their amino-group contents when M_{lim} values were adjusted to 2.0×10^3. As shown in Table 1, the activity of the PL-cellulose adsorbent is strongly dependent on the M_{lim} value when the amino-group content is 0.6 meq/g: the endotoxin-adsorbing capacity increased from 185 to 480 μg per ml of wet adsorbent and the $K_{d,app}$ decreased from 3.8×10^{-10} to 1.1×10^{-11} *M*, while the M_{lim} increased from 2.0×10^3 to $>2\times10^6$. The smaller the $K_{d,app}$ of endotoxin to the adsorbent is, the stronger the endotoxin-removing activity of the adsorbent.

As a result, it was found that PL-cellulose (10^6), having the largest M_{lim} of $>2\times10^6$, showed the greatest endotoxin-removing activity. The $K_{d,app}$ of PL-cellulose [62] and cross-linked DMP [61] particles are respectively about 1/13 and 1/18, as large as that of polymyxin-Sepharose, although each had the smallest M_{lim} of 2×10^3. Minobe et al. found that the endotoxin-adsorbing capacity and $K_{d,app}$ values of histidine-Sepharose were 530 μg per ml of wet adsorbent and 1.57×10^{-9} *M*, at pH 7.0 and $\mu=0.02$, respectively [44]. As a result, the LPS-removing activities of PL-cellulose and cross-linked DMP are superior to that of polymyxin-Sepharose and histidine-Sepharose.

To achieve selective removal of endotoxins, it is important to check adsorbing activity for proteins. Fig. 6 shows the effect of the adsorbent pore size (M_{lim}) on adsorption of endotoxin, BSA (basic protein) and γ-globulin (hydrophobic protein) by PL-cellulose adsorbents. As a result, the adsorption of all cellular products increased with increasing

Table 1
Effect of adsorbent's pore size on adsorption of LPS (modified after Sakata et al. [51])

Adsorbent					Adsorbing capacity[c] of endotoxin (μg/ml adsorbent)	$K_{d,app}$[d] of endotoxin (*M*)
Name	Matrix	Ligand	Pore size of matrix (M_{lim}[a])	Amino-group content[b] (meq/g)		
PL-cellulose(10^3)[e]	Cellufine-GC15	Poly(ε-lysine)	2×10^3	0.6	185	3.8×10^{-10}
PL-cellulose(10^4)[e]	Cellufine-GC700	Poly(ε-lysine)	1×10^4	1.4	280	5.5×10^{-11}
PL-cellulose(10^6)[e]	Cellufine-CPC	Poly(ε-lysine)	$>2\times10^6$	0.6	480	1.1×10^{-11}
Polymyxin-Sepharose[f]	Sepharose	Polymyxin B	$>2\times10^6$	0.2	250	1.1×10^{-9}
Cross-linked DMP[g]	(Copolymer) DMP/BBMA		2×10^3	4.1	360	7.2×10^{-11}

[a] M_{lim} (expressed as polysaccharide molecular mass) was calculated by means of calibration curves [63] of size-exclusion chromatography obtained from aqueous solution.

[b] Content of amino groups in the adsorbent.

[c,d] The endotoxin-adsorption capacity per ml adsorbent and the apparent dissociation constant ($K_{d,app}$) of endotoxin to adsorbent were estimated by an adsorption isotherm, as described previously [44]. The adsorption isotherm of endotoxin was determined using a batchwise method with 0.1 ml of wet adsorbent and 4 ml of an endotoxin solution (LPS from *E. coli* O111:B4 (M_n 1×10^6): 1×10^4 to 2×10^6 EU/ml, pH 7.0, $\mu=0.05$). The $K_{d,app}$ was expressed in mol/l (*M*) of molecular mass of endotoxin.

[e] Poly(ε-lysine)-immobilized cellulose spherical particles [62].

[f] Detoxi-Gel [37,38].

[g] Spherical copolymers of *N*,*N*-dimethylaminopropylacrylamide (DMP) and *N*,*N*-butylene-bis-methacrylamide (BBMA) [61].

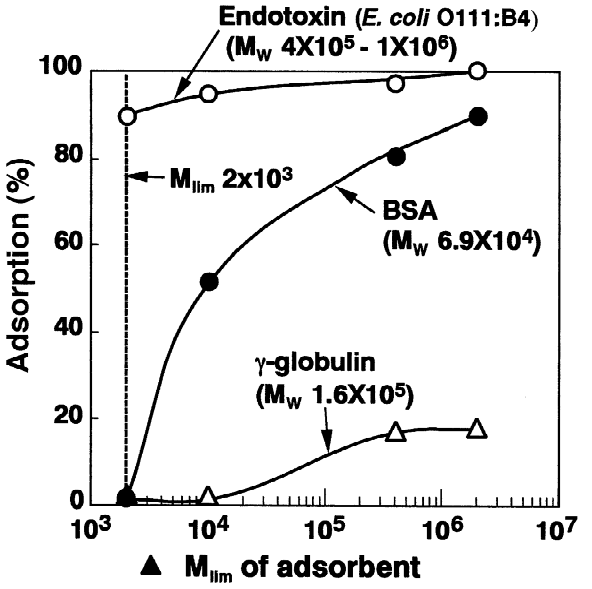

Fig. 6. Effect of an adsorbent's pore-size on the adsorption of cellular product. The adsorption of a cellular product was determined by a batchwise method with 0.2 ml of wet PL-cellulose adsorbent (diameter, 44 to 105 μm; amino-group content, 0.6 meq/g; M_{lim} 2×10^3–>2×10^6) and 4 ml of a sample solution (100 μg/ml, pH 7.0, ionic strength $\mu=0.05$) (examined in our laboratory). Pore size of the adsorbent (M_{lim} expressed as polysaccharide molecular mass) was calculated by means of calibration curves of size-exclusion chromatography obtained from an aqueous solution [57].

M_{lim} of the adsorbent from 2×10^3 to $>2\times10^6$. Only when the PL-cellulose, with an M_{lim} of 2×10^3, was used as the adsorbent, was the endotoxin adsorbed without adsorption of BSA and γ-globulin. In addition, other neutral or basic proteins were almost not adsorbed to the adsorbents under similar conditions.

The result (Fig. 6) showed that the adsorption of protein was caused mainly by the entry of the protein into the pores of the adsorbent. This indicates that both BSA (M_w 6.9×10^4) and γ-globulin (M_w 1.6×10^5) can readily penetrate into a particle with an M_{lim} of 2×10^6, but cannot penetrate into a particle with an M_{lim} of 2×10^3. On the other hand, it would also appear that endotoxin aggregates cannot enter the pores of adsorbents with an M_{lim} of 2×10^3 because its molecular mass (M_w 4×10^5 to 1×10^6 being supramolecular assemblies) [57] is remarkably larger than the M_{lim} of the adsorbent. Much of the standard endotoxin (LPS from *E. coli* O111:B4), however, was well adsorbed even by the adsorbent with an M_{lim} of 2×10^3, as shown in Table 1 and Fig. 6. We previously reported [57] that the endotoxin molecules were adsorbed by aminated PMLG particles not only into the pores of the particles but also on their surfaces. PL-cellulose particles have the same characteristics. By contrast, polymyxin-Sepharose showed the weakest endotoxin-removing activity ($K_{d,app}$, 5.0×10^{-9} M) of all the adsorbents, in spite of it having the largest M_{lim} of $>2\times10^6$. This weak LPS-removing activity is probably due to it having the smallest amino-group content (0.2 meq/g). The results indicate that proper selection of M_{lim} of the adsorbent enables the selective removal of endotoxin from acidic or hydrophobic protein-containing solutions.

3.2.2. Effects of ionic and hydrophobic interaction. The effects of a buffer's ionic strength and its pH on adsorption of endotoxin by the various adsorbents were examined. As shown in Fig. 7a, the higher the ionic strength of the buffer, the lower the LPS-adsorbing activity of all cationic adsorbents. By contrast, the activity on Cellufine-GC15 (non-immobilized cellulose particles), which was used as the matrix of PL-cellulose(10^3), increased from 17 to 38% with an increase of ionic strength from $\mu=0.05$ to 1.0. However, both PL-cellulose(10^6) and (10^3) always showed a greater endotoxin-adsorbing activity (99 to 92%) at a wide ionic strength of $\mu=0.05$–0.8. The adsorbing activity of polymyxin-Sepharose and cross-linked DMP decreased markedly when the ionic strength was increased to 0.4 or higher. At a wide pH range of 4.0–9.0 and an ionic strength of $\mu=0.05$, PL-cellulose(10^6) with the largest pore size always showed the highest LPS-adsorbing activity (>98%) (Fig. 7b). PL-cellulose(10^3) also showed a high activity (>98%) at pHs of 6.0 to 9.0, although it decreased from 99 to 87% as the pH range decreased from 6.0 to 4.0. On the other hand, polymyxin-Sepharose showed high adsorbing activity only at pH 7.0. Cross-linked DMP also showed high activities at pHs from 6.0 to 9.0, similarly to PL-cellulose(10^3).

From these results (Fig. 7a and b) we assumed that the LPS-adsorbing activity of PL-cellulose was due to the simultaneous effects of the cationic properties originating from ligands, and the hydrophobic or other properties originating from the matrix. The

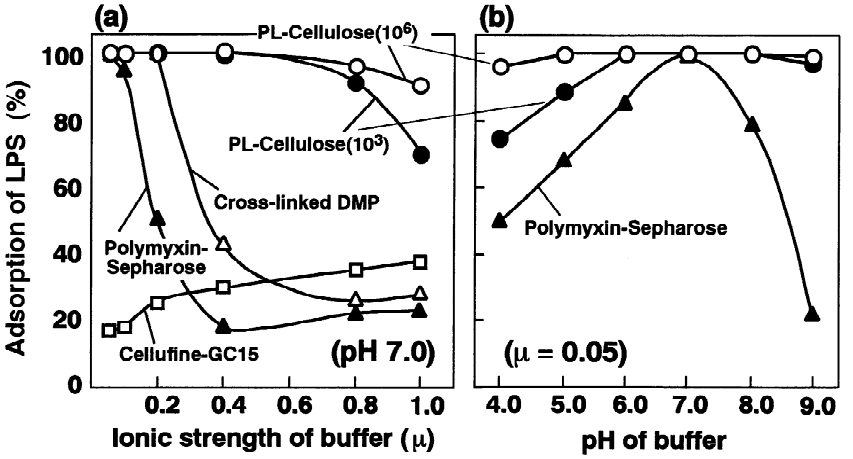

Fig. 7. Effects of buffer's (a) ionic strength and (b) its pH on the adsorption of endotoxin by various adsorbents (modified after Todokoro et al. [62]). The adsorption of endotoxin was determined using a batchwise method with 0.2 g of the wet adsorbent and 2 ml of an endotoxin solution (LPS (*E. coli* O111:B4), 1 μg/ml). Cellufine-GC15 particles: matrix before introduction of poly(ε-lysine).

adsorbing activity of PL-cellulose, being dependent on the ionic strength of the buffer and its pH values, suggests a cationic interaction. Further, the LPS-adsorbing activity of non-immobilized cellulose particles (the matrix of PL-cellulose(10^3)) increased with an increase of the buffer's ionic strength (Fig. 7a). Matsumae et al. [45] reported that a hydrophobic bond was formed between LPS and histidine-immobilized Sepharose under conditions of high ionic strengths. These results suggest that PL-cellulose particles also adsorb endotoxin by a hydrophobic property that originates from the matrix.

It seems that the adsorption of the protein is also induced by both cationic and hydrophobic properties. Matsumae et al. [45] described that acidic or neutral substances are adsorbed to histidine-immobilized Sepharose at a pH more basic than the isoelectric point of the substance under a condition of $\mu = 0.02$. Polymyxin-Sepharose and PL-cellulose also have the same characteristics. The charge of BSA is anionic at pH values greater than its p*I* (4.9); also the adsorption of BSA by the adsorbents is dependent on the ionic strength, as shown in Fig. 8a. This suggests ionic interaction between the adsorbent and BSA. On the other hand, γ-globulin (p*I* 7.4) is a neutral and weakly hydrophobic protein, and its adsorption is independent of ionic strength, as shown in Fig. 8b. These findings suggest the participation of hydrophobic binding.

These results (Figs. 6–8) show that the cationic adsorbents bind more strongly with endotoxin than BSA or γ-globulin. This is because endotoxin has a lower pK_a and a higher hydrophobicity than each protein. The PL-cellulose with small pore size of M_{lim} of 2×10^3 adsorbed little of each protein. That is to say, the ionic and hydrophobic interaction of protein is caused mainly by the entry of the protein into a pore of an adsorbent with M_{lim} over 2×10^3. The results also indicate that removal using more hydrophobic adsorbents is less dependent on the pH and ionic strength. However, the stronger the hydrophobicity of the adsorbent, the stronger the binding activity of hydrophobic protein, such as γ-globulin, by the adsorbent. This suggests that it is also effective to decrease the hydrophobicity of adsorbent by increasing its cationic activity for the selective removal of endotoxin from hydrophobic protein-containing solutions. Indeed, incorporation of amino groups into the PMLG adsorbent successfully decreases the hydrophobic interaction with hydrophobic protective antigen of *B. pertussis*, and increases the adsorption of its endotoxin at the same time [56].

3.2.3. Simultaneous effect of various factors on the selective removal of endotoxin

For the selective removal of LPS from a protein solution, it is also necessary to adjust buffer con-

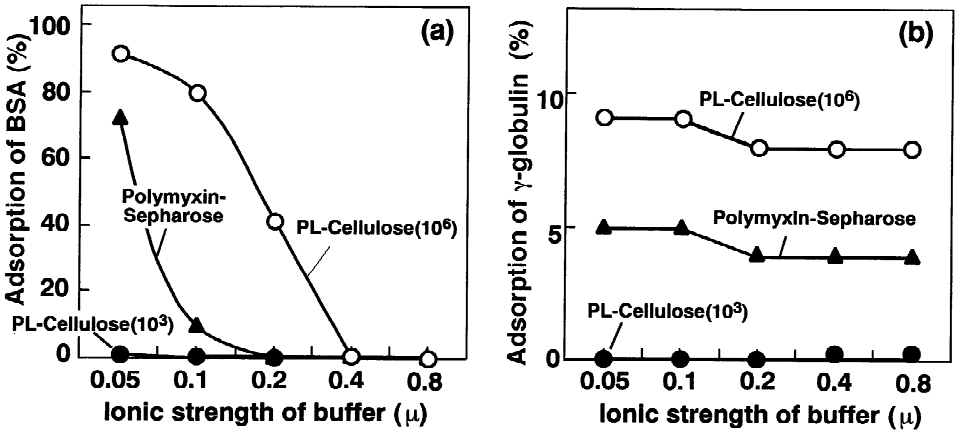

Fig. 8. Effect of a buffer's ionic strength on adsorption of (a) BSA and (b) γ-globulin by various adsorbents (modified after Todokoro et al. [62]). The adsorption of endotoxin was determined using a batchwise method with 0.2 g of the wet adsorbent and 2 ml of a protein solution (500 μg/ml, pH 7.0, and ionic strength of $\mu = 0.05–0.8$).

ditions such as ionic strength and pH. The effects of ionic strength on the selective adsorption of LPS from a BSA-containing solution with various adsorbents were examined (results are shown in Fig. 9a–f). When a BSA solution, 500 μg/ml of BSA and 100 ng/ml of standard LPS, was used as a sample solution, PL-cellulose(10^3) selectively adsorbed LPS in the solution at an ionic strength of $\mu = 0.05$ to 0.4 and pH 7.0, without adsorption of BSA (Fig. 9a). Cross-linked DMP and aminated PMLG showed a high endotoxin selectively at $\mu = 0.05$ to 0.2 (Fig. 9b) and 0.2 to 0.4 (Fig. 9c), respectively. PL-cellulose(10^6) selectively adsorbed LPS only at $\mu = 0.4$ (Fig. 9d). By contrast, polymyxin-Sepharose and histidine-Sepharose showed adsorbing activities for both LPS and BSA at a low ionic strength of $\mu =$ 0.05 to 0.1, and the adsorbing activities decreased with an increase in the ionic strength (Fig. 9e and f, respectively). Each adsorbent therefore cannot selectively adsorb endotoxin from the BSA solution at all ionic strengths.

The endotoxin-removing activity of PL-cellulose(10^3) was compared with that of PL-cellulose(10^6) and cross-linked DMP, and the results are shown in Table 2. Various protein solutions, which were naturally contaminated with LPS at concentrations from 15 to 320 EU/ml, were used as samples. It is essential to reduce endotoxin to a concentration at least lower than 1 EU/ml in fluids used for intravenous injection, so as not to elicit pyrogenic reactions in mammals [44]. As shown in Table 2, when PL-cellulose(10^3), (10^6) and cross-linked DMP were used as adsorbent at ionic strengths of $\mu = 0.05$, 0.4 and 0.05, respectively, all adsorbents were able to remove LPS from all protein to a level below 1 EU/ml. PL-cellulose(10^6) having a larger pore size always showed higher removing activities: the residual concentrations of endotoxin after treatment were less than 0.1 EU/ml in all cases.

4. Discussion

In order to achieve a selective removal of endotoxin from final biological products, such as protein and protective antigen, by using adsorbent, it is necessary to consider not only the chemical and physical structures of endotoxin but also those of adsorbents and proteins, and the solution conditions. In physiological solutions, endotoxin aggregates form supramolecular assemblies (M_w 4×10^5 to 1×10^6) with phosphate groups as the head group, and exhibit a net-negative charge because of their phosphate groups. However, since proteins may release endotoxin monomers from the aggregates [31], we assume that endotoxin aggregates form in wide molecular sizes of M_w from 2×10^4 to 1×10^6 in physiological solutions (Fig. 10). On the other hand, the molecular masses of proteins are generally about 1×10^4 to 5×10^5. Therefore, it is extremely difficult to separate endotoxin from protein only by size-

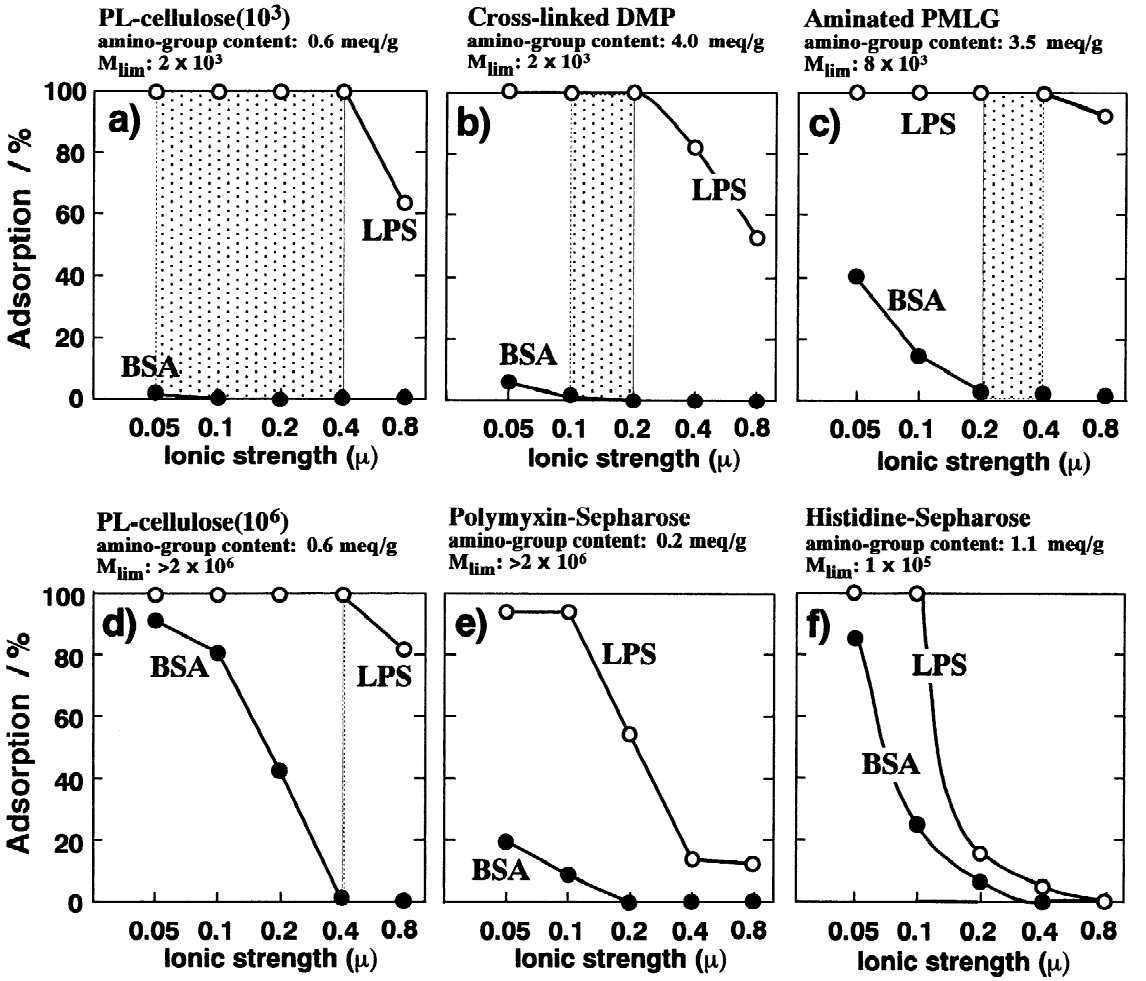

Fig. 9. Effects of ionic strength on selective adsorption of endotoxin from a BSA solution containing LPS by the various cationic adsorbents. The selective adsorption of endotoxin was determined by a batchwise method with 0.2 g of the wet adsorbent and 2 ml of a sample solution (BSA 500 μg/ml, LPS (*E. coli* O111:B4) 100 ng/ml, pH 7.0, and ionic strength of $\mu = 0.05–0.8$).

separation methods, such as size-exclusion chromatography and ultrafiltration.

The charge of the protein is anionic at a pH over p*I* and cationic at a pH under it. Accordingly, the most favorable pH for the selective removal of endotoxin from a protein solution is one below its p*I* and above the pK_a (pK_1 1.3 [26]) of the phosphate residues of the endotoxin. Most cationic adsorbents, such as DEAE-Sepharose, histidine-Sepharose, readily removed endotoxin from cytochrome *c* (a basic protein) and γ-globulin (a neutral protein) solutions at pH 7 without a loss of protein [44,57,60]. This is because the ionic interaction of the adsorbent with cytochrome *c* (p*I* 10.6) and γ-globulin (p*I* 7.4) is not induced at pH 7.0 (lower than p*I*). In contrast, high-molecular mass acidic substances such as BSA are anionic at pH 7.0 (over p*I*), and are considerably adsorbed by cationic adsorbents. To selectively adsorb endotoxin in a BSA solution, it is necessary to decrease the pH value of the buffer to 4.9 (p*I* of BSA) or lower, or increase the ionic strength (μ) of buffer to 0.2 or higher, but at such a low pH, the endotoxin-adsorbing activity of the adsorbent decreases (Fig. 7b). Therefore, the development of adsorbents capable of retaining high endotoxin selectivity, which is less dependent on pH and ionic strength, is keenly pursued.

The present results (Table 2) suggest that PL-

Table 2
Selective removal of endotoxin from a protein solution by PL-cellulose(10^3), (10^6) and cross-linked DMP adsorbents (modified after Sakata et al. [51])

Sample			PL-cellulose(10^3) ($\mu=0.05$, pH 7.0)		PL-cellulose(10^6) ($\mu=0.40$, pH 7.0)		Cross-linked DMP ($\mu=0.05$, pH 7.0)	
Compound (1 mg/ml)	p*I*	Conc. of natural endotoxin (EU/ml)	Conc. of remaining endotoxin (EU/ml)	Protein recovery (%)	Conc. of remaining endotoxin (EU/ml)	Protein recovery (%)	Conc. of remaining endotoxin (EU/ml)	Protein recovery (%)
Ovalbumin	4.6	280	0.81	99	<0.10	95	0.85	98
BSA	4.9	320	0.45	99	<0.10	97	0.55	97
Myoglobin	6.8	45	0.18	99	<0.10	98	<0.10	99
γ-Globulin	7.4	56	0.20	99	<0.10	97	0.34	98
Cytochrome *c*	10.6	15	0.15	99	<0.10	98	<0.10	99

The removal of endotoxin was determined using a batchwise method with 0.3 ml of wet adsorbent and 2 ml of a protein solution (1 mg/ml) containing natural endotoxin.

cellulose and cross-linked DMP spherical particles can reduce the concentrations of natural endotoxins to 0.1 EU/ml or lower in drugs and fluids used for intravenous injection, at a neutral pH and ionic strengths of $\mu=0.05$ to 0.4. These processes did not affect the recovery of even acidic proteins such as BSA. As shown in Fig. 10, the high endotoxin-adsorbing activity of the particles is possibly due to the cationic properties of the ligand and the suitable hydrophobic properties of the matrix. The high endotoxin selectivities of the particles with small pore sizes are due to the size-exclusion effects on protein molecules. By contrast, those of the particles with large pore sizes are due to the decreases of ionic interaction for net-negative charged proteins, which arise when the buffer's ionic strength is adjusted to 0.2 or stronger.

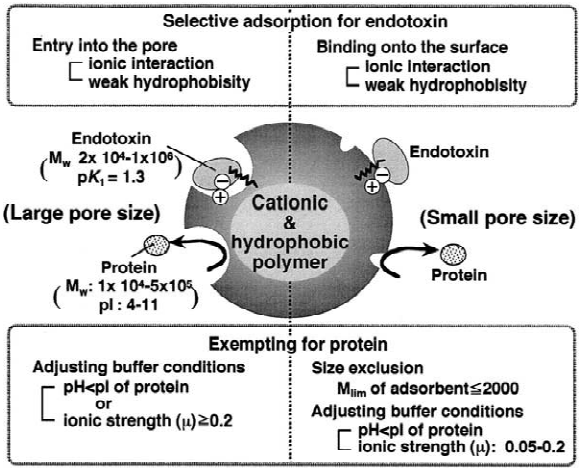

Fig. 10. Several factors for the selective removal of endotoxins from protein solutions by cationic and hydrophobic polymers.

In view of the adsorbing capacity of endotoxins, the cationic polymers having large pore sizes show greater capacity, because of the entry of endotoxin molecules into the large pores. Thus these are suitable as adsorbent for removal of endotoxin from bioproducts containing large quantities of endotoxin, such as a crude antigen solution originating from a gram-negative bacterium.

For practical application, ease of regeneration is very important. The cross-linked DMP and PL-cellulose spherical particles can be completely regenerated by frontal chromatography with 0.2 *M* sodium hydroxide followed by 2.0 *M* sodium chloride. Their stable structures, resisting extreme pH values, are due to their –CONH– and –CHNH– bonds, respectively. In addition, we believe that the cross-linked DMP and PL-cellulose particles are better column packing materials for endotoxin removal, because of their higher flow-rate resistance than that of conventional polysaccharide gels.

Of course, the development of even better adsorbents should be pursued by continuing this search for materials. In order to achieve selective removal of endotoxins, it is important to not only select suitable ligands but also adjust the pore size of the matrix.

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