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Journal of Chromatography A, 676 (1994) 267–275

JOURNAL OF CHROMATOGRAPHY A

Cross-linked N,N-dimethylaminopropylacrylamide spherical particles for selective removal of endotoxin

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First received 30 November 1993; revised manuscript received 14 March 1994

Abstract

Spherical polymer adsorbents for endotoxin adsorption were prepared by suspension copolymerization of N,N-dimethylaminopropylacrylamide (DMAPAA) with N-allylacrylamide (AAA). The amino-group contents and the pore size of the adsorbents were easily adjusted by changing the monomer ratio and diluent ratio. The more amino groups are introduced, the larger is the endotoxin-adsorbing capacity of the adsorbent, and the smaller the pore size (molecular mass exclusion, M_{lim}) of the adsorbent, the less acidic proteins such as bovine serum albumin are adsorbed. When M_{lim} was smaller than 300 (as the molecular mass of polysaccharide) and the amino group content was 4.5 mequiv./g, the DMAPAA–AAA adsorbent showed a high endotoxin-removing activity at an ionic strength of $\mu = 0.05$–0.4 and pH 5–9. The adsorbent was also able to remove endotoxin from a protein solution, naturally contaminated with endotoxin, at $\mu = 0.05$ without affecting the recovery of the protein. The adsorbent can be completely regenerated by washing with 0.2 *M* sodium hydroxide followed by 2.0 *M* sodium chloride.

1. Introduction

Removal of endotoxin (lipopolysaccharide, LPS) from substances used as drugs is very important, as its potent biological activity causes pyrogenic and shock reactions in mammals on intravenous injection even in nanogram amounts [1–3]. Endotoxin, a constituent of the cell wall of Gram-negative bacteria, is a potential contaminant of physiological fluids and aqueous solutions and a very stable molecule which resists extreme temperatures and pH values. For removing endotoxin from solutions of high-molecular-mass compounds, such as proteins, adsorption techniques are used.

Recently, some endotoxin adsorbents, immobilized histidine [4–6] and polymyxin-Sepharose [7], have become commercially available. Although these adsorbents have high capacities for adsorbing endotoxin, adsorption of acidic proteins such as bovine serum albumin (BSA) also occurs at a low ionic strength of $\mu = 0.05$ and neutral pH. As the ionic strength increased, the adsorption of BSA by the adsorbents decreased, and at a high ionic strength ($\mu = 0.2$–0.8) adsorption of endotoxin was also very slight [5].

We attempted, therefore, to develop endotoxin adsorbents capable of retaining a high endotoxin-removing activity over a wide range of ionic strength. We have reported that aminated poly(γ-methyl L-glutamate) (PMLG) spheres

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0021-9673/94/$07.00
SSDI 0021-9673(94)00264-A

having diaminoethane as a ligand have a high endotoxin-removing activity even at a high ionic strength ($\mu = 0.2$–0.8) [8,9]. Further, we found that the interaction of various proteins with the aminated PMLG adsorbent decreased with decrease in the pore size of the spheres [10]. However, this adsorbent is unsatisfactory with respect to complete regeneration because it is generally considered that PMLG, having esteratic sites (–CO–O– bonds), is gradually hydrolysed in an alkaline solution, one of the solvents used for regeneration.

In this work, we attempted to develop novel endotoxin adsorbents that can be produced on a large scale and can be easily regenerated many times. This paper describes the synthesis of novel spherical copolymers from N,N-dimethylaminopropylacrylamide (DMAPAA) (monomer), having amino groups [CH_2=CHCONH(CH_2)$_3$ · N(CH_3)$_2$], and N-allylacrylamide (AAA) (water-soluble cross-linking agent) (CH_2=CHCONHCH$_2$CH=CH_2), being highly soluble in the monomer by one-step polymerization. We describe also the characteristics and applications of the spherical copolymers for the selective removal of endotoxin and the regeneration of this adsorbent.

2. Experimental

2.1. Materials

Purified endotoxin (*Escherichia coli* UKT-B) was purchased from Wako (Osaka, Japan) and other endotoxins from Difco (Detroit, MI, USA). Limulus ES-J test Wako (Limulus amoebocyte lysate) was a product of Wako.

Immobilized histidine (Pyro Sep) was purchased from Daicel (Tokyo, Japan). N,N-Dimethylaminopropylacrylamide (DMAPAA) monomer (Kohjin, Tokyo, Japan) and N-allylacrylamide (AAA) cross-linking agent (Kohjin) were purified by vacuum distillation at 131°C/1 mmHg and 115°C/0.7 mmHg, respectively.

Proteins were purchased from Wako. Endotoxin-free water was kindly supplied by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). All other chemicals were of analytical-reagent grade.

2.2. Synthesis of adsorbents

DMAPAA monomer, AAA as a cross-linking agent, 1-hexanol as a diluent and 2 mass-% azobisisobutyronitrile as an initiator were mixed at room temperature. The mixture was added to a 25 mass-% anhydrous sodium sulphate solution containing 1% sodium carboxymethylcellulose, which were suspended by stirring. The suspension was heated at 80°C for 12 h. The DMAPAA–AAA (DAA) copolymer particles obtained were washed successively with water, hot water, methanol and ethanol.

DAA particles with diameters of 44 to 105 μm were used as adsorbents.

2.3. Determination of amino group contents of adsorbents

Amino groups were determined by pH titration and elemental analysis as described previously [8].

2.4. Determination of the pore size of the adsorbent

The prepared DAA adsorbents were packed into a stainless-steel column (150 × 5 mm I.D.). The chromatograph included a JASCO Model 880-PU pump and a Shodex SE-51 refractometric monitor. The pore size (molecular mass exclusion, M_{lim}) of the matrix in the adsorbent was calculated from calibration graphs obtained by size-exclusion chromatography (SEC). Homogeneous series of pullulan and maltose were used as permeable substances. The calibration graphs were obtained by plotting the average molecular masses against the peak elution volumes. The M_{lim} value was determined by extrapolating the linear part of the graph as described previously [11,12].

2.5. Adsorption of endotoxin

The adsorption of endotoxin was measured by a batchwise method as follows. The adsorbent was washed and equilibrated with various buffers with different ionic strengths as described previously [9]. A 0.2–0.3-g portion of wet adsorbent was suspended in 2–3 ml of an endotoxin solution. The suspension was shaken for 2 h at 25°C and filtered through a Millipore filter (0.8 μm) to remove the adsorbent. The endotoxin content of the filtrate was determined. The apparent dissociation constant (K_d) between endotoxin and adsorbent was calculated from the adsorption isotherm as described previously [5,6].

2.6. Reproducibility of adsorbent

The reproducibility of the adsorbent was determined with a frontal column as follows. A 0.7-ml portion of wet adsorbent was packed in a sterilized glass column (10 × 0.3 cm I.D.) and the column was washed with 20 ml of 2.0 *M* sodium chloride and then equilibrated with 0.02 *M* phosphate buffer (pH 7.0, $\mu = 0.05$). An endotoxin solution (1000 ng/ml, purified LPS from *E. coli* UKT-B) was passed through the column at a flow-rate of 0.2 ml/min at room temperature. Fractions of 25 ml were collected and the endotoxin concentration in each fraction was determined.

The column was reused after washing with 20 ml of 0.2 *M* sodium hydroxide, 2.0 *M* sodium chloride and endotoxin-free water.

2.7. Endotoxin assay

Endotoxin was assayed by the Limulus test involving turbidimetric time assay at 660 nm with a Toxinometer ET-201 (Wako) [13]. Purified endotoxin (*E. coli* UKT-B) was used as the standard. Limulus ES-J test Wako was used as the reagent for the reaction.

2.8. Protein assay

The protein concentration was measured with a UV-160 spectrophotometer (Shimadzu) at 280 nm (proteins other than cytochrome *c*) or 410 nm (cytochrome *c*).

3. Results and discussion

3.1. Effects of various factors on the endotoxin-adsorbing activity of adsorbents

The endotoxin-adsorbing activities of the adsorbents were examined by the batchwise method with various kinds of buffers. Two kinds of standard LPS, from *E. coli* UKT-B and O111:B4, were used as endotoxin-containing samples.

The effects of amino-group contents or M_{lim} of DMAPAA–AAA (DAA) adsorbents on the adsorption of endotoxins were investigated at pH 7.0 and ionic strength $\mu = 0.05$. The results are given in Table 1. DAA adsorbents with pore sizes <300–40 000 as M_{lim} and amino-group content [$-N(CH_3)_2$] of 1.1–5.1 mequiv./g were prepared. The amino-group content and M_{lim} of the adsorbents were easily adjusted by changing the DMAPAA (monomer) ratio and 1-hexanol (diluent) ratio in the suspension copolymerization; the amino-group content increased from 1.1 to 5.1 mequiv./g with increase in the monomer ratio from 10 to 90 mol-%, and M_{lim} increased from <300 to 45 000 with increase in the diluent ratio (to the DMAPAA–AAA solution) from 0 to 200 vol.-%. Each adsorbent satisfactorily adsorbed endotoxin from endotoxin solutions. When the amino-group content of the adsorbent was 4.5–5.1 mequiv./g (DAA-70–0, -90–0, -70–100, -70–200), the residual concentration of each endotoxin after treatment was below 10 pg/ml.

Fig. 1 shows an electron micrograph of a typical example of DAA adsorbents prepared from DMAPAA and AAA by one-step polymerization without a diluent. The adsorbents were spherical particles with a diameter of about 100 μm.

For selective adsorption of endotoxin, it is necessary to reduce cationic and hydrophobic interactions of the adsorbent with substances such as proteins. The effects of the ionic strength and M_{lim} of the adsorbent on the adsorption of

Table 1
Adsorption of endotoxin by various DAA adsorbents

Adsorbent							Residual concentration of endotoxin after treatment (pg/ml)	
Type	Molar ratio		Diluent[a] (%, v/v)	M_{lim}[b]	Amino-group content[c] (mequiv./g)	S_d[d] (wet-ml/dry-g)		
	DMAPAA (mol%)	AAA (mol%)					*E. coli* UKT-B	*E. coli* O111:B4
DAA-10–0	10	90	0	<300	1.1	2.2	350 000	174 000
DAA-30–0	30	70	0	<300	2.3	2.6	175 000	65 000
DAA-50–0	50	50	0	<300	3.8	2.8	50 000	150
DAA-70–0	70	30	0	<300	4.5	4.3	<10	<10
DAA-90–0	90	10	0	<300	5.1	7.9	<10	<10
DAA-70–100	70	30	100	10 000	4.4	9.0	<10	<10
DAA-70–200	70	30	200	43 000	4.5	23.8	<10	<10

[a] % (v/v) of *n*-hexanol to DMAPAA–AAA solution.
[b] Value reduced as molecular mass of polysaccharide.
[c] Amino groups introduced into the adsorbent.
[d] Degree of swelling in water.
[e] Adsorption of endotoxin by the adsorbent was determined by the batchwise method with 0.3 g of wet adsorbent and 3 ml of endotoxin solution (*E. coli* LPS, 500 ng/ml, pH 7.0, $\mu = 0.05$).

BSA, an acidic protein, were examined with various adsorbents at ionic strengths $\mu = 0.05$–0.4. The adsorbents with an amino-group content of 4.5 mequiv./g and M_{lim} of <300–43 000 were used and the results are shown Fig. 2. Little BSA was adsorbed by the DAA-70–0 adsorbent ($M_{lim} < 300$) at any ionic strength. The BSA-adsorbing capacity of the other adsorbents decreased with increase in the ionic strength. At a low ionic strength ($\mu = 0.05$), the adsorption rate of BSA increased from 0 to 98% with increase in M_{lim} of the adsorbent from <300 (DAA-70–0) to 43 000 (DAA-70–200). Im-

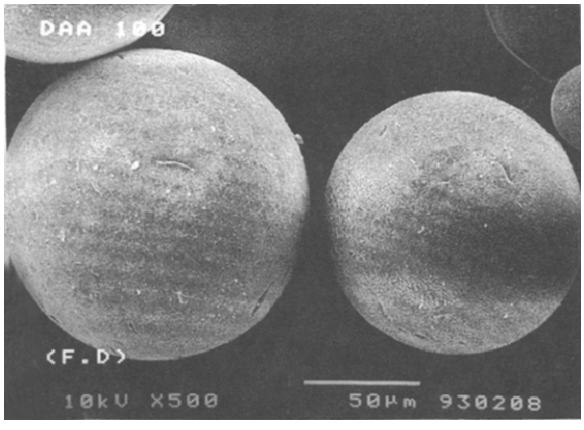

Fig. 1. Electron micrograph of DAA adsorbents prepared without a diluent. The amino-group content of adsorbents was 4.5 mequiv./g. The scale bar represents 50 µm.

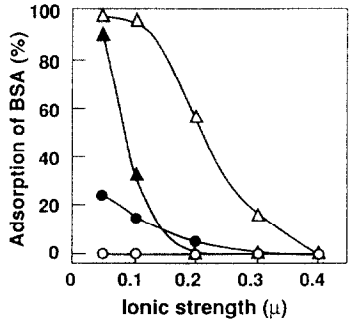

Fig. 2. Effects of ionic strength and M_{lim} on adsorption of BSA by various adsorbents. The adsorption of BSA was determined by the batchwise method with 0.3 g of each wet adsorbent and 3 ml of a BSA solution (500 µg/ml, pH 7.0, $\mu = 0.05$–0.4). Adsorbent, M_{lim}: (○) DAA-70–0, <300; (●) DAA-70–100, 10 000; (△) DAA-70–200, 43 000; (▲) immobilized histidine, 100 000. Amino-group contents: DAA adsorbents, 4.5 mequiv./g; immobilized histidine, 0.3 mequiv./g.

mobilized histidine with a large pore size (M_{lim} 100 000) also showed high adsorption of BSA at a low ionic strength of $\mu = 0.05$. The other DAA (DAA-10–0, -30–0, -50–0, -90–0) adsorbents with $M_{lim} < 300$ hardly adsorbed BSA, just like DAA-70–0. Little γ-globulin (a neutral protein) or cytochrome *c* (a basic protein) was adsorbed by any adsorbent under any conditions (data not shown).

These results suggest that DAA-10–0, -30–0, -50–0, -70–0 and -90–0 can adsorb endotoxins without affecting the recovery of the proteins. However, DAA-10–0, -30–0 and -50–0 are unsatisfactory with respect to endotoxin-adsorbing capacity and DAA-90–0 is disadvantageous with a high flow-rate in the chromatographic process because of its high degree of swelling (S_d) of 7.8, as shown in Table 1. In contrast, the DAA-70–0 adsorbent with an S_d of 4.3 (similar to that of an aminated PMLG sphere [14]) is expected to show a higher flow-rate resistance than Sepharose gels.

The adsorption isotherms for DAA-70–0 (amino-group content 4.5 mequiv./g) and immobilized histidine (amino-group content 0.3 mequiv./g) adsorbents were determined in phosphate buffer (pH 7.0, $\mu = 0.05$) by changing the concentration of endotoxin (*E. coli* O111:B4 LPS) with the batchwise method. The endotoxin adsorption of each adsorbent increased with increasing concentration of endotoxin. Fig. 3 shows the Scatchard plots [6] derived from these adsorption isotherms. According to these plots, the adsorption capacities and dissociation constants (K_d) were calculated to be 0.81 mg endotoxin/g wet adsorbent and $8.5 \cdot 10^{-10}$ *M* in the DAA-70–0 adsorbent and 0.21 mg and $1.1 \cdot 10^{-9}$ *M* in the immobilized histidine, when the aggregation mass of endotoxins was estimated as 10^6. The adsorption capacity of the DAA-70–0 adsorbent was about four times larger than that of the immobilized histidine although both of the adsorbents showed similar K_d values. These results indicate that a high amino-group content increased substantially the endotoxin-adsorbing activity of the DAA-70–0 adsorbent.

The adsorption of endotoxins originating from various Gram-negative bacteria by the DAA-70–0 adsorbent were investigated, and the results are given in Table 2. When adsorption of endotoxin was measured in water, the DAA-70–0 adsorbent showed a high endotoxin-adsorbing activity for various purified endotoxins. Under the conditions of pH 7.0 and $\mu = 0.1$, the residual concentrations of endotoxin originating from *E. coli* UKT-B and O111:B4 decreased

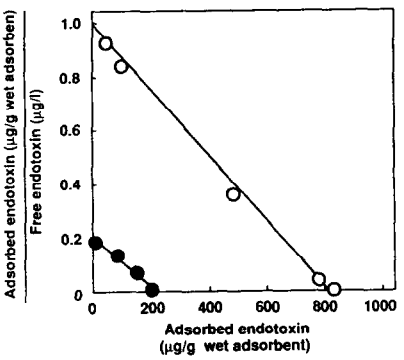

Fig. 3. Scatchard plots derived from adsorption isotherms of endotoxin on (○) DAA-70–0 adsorbent and (●) immobilized histidine. The adsorption of endotoxin was determined by the batchwise method with 0.2 g of the wet adsorbent and 2 ml of an endotoxin solution (*E. coli* O111:B4 LPS, 10^3–10^6 ng/ml, pH 7.0, $\mu = 0.05$).

Table 2
Adsorption of endotoxins originating from various Gram-negative bacteria by DAA-70–0 adsorbent

Endotoxin[a]	Residual concentration of endotoxin after treatment[b] (pg/ml)	
	Water	Buffer (pH 7.0, $\mu = 0.1$)
Escherichia coli UKT-B	<10	<10
Escherichia coli O111:B4	<10	<10
Escherichia coli O127:B8	<10	5600
Escherichia coli O55:B5	<10	100
Salmonella typhosa O901	<10	50
Salmonella typhimurium	<10	600
Bordetella pertussis Tohama	<10	80

[a] The purified endotoxin was dissolved in water and phosphate buffer (pH 7.0, $\mu = 0.1$) at a concentration of 500 ng/ml.

[b] The adsorption of endotoxin was determined by the batchwise method with 0.3 g of wet adsorbent and 3 ml of an endotoxin solution.

from 500 to less than 10 pg/ml, but all other endotoxins were not completely removed.

The effects of pH and ionic strength on the removal of endotoxin by DAA-70–0 and immobilized histidine were investigated in various kinds of buffers and the results are shown in Fig. 4a and b. Each residual concentration of endotoxin increased with increasing ionic strength at any pH. When the ionic strength was adjusted to $\mu = 0.05$, both adsorbents removed endotoxin satisfactorily at around neutral pH. When the ionic strength was increased ($\mu = 0.1$–0.4), the endotoxin-removing activity of immobilized histidine decreased considerably at any pH value (Fig. 4b). DAA-70–0 adsorbent was able to decrease the residual concentration of endotoxin from 100 ng/ml to less than 100 pg/ml at μ = 0.05–0.2 and pH 6–8 (Fig. 4a).

The effects of pH on the removal of endotoxin from a BSA solution containing endotoxin (endotoxin 100 ng/ml as *E. coli* O111:B4 LPS, BSA 500 μg/ml) with DAA-70–0 adsorbent or immobilized histidine were examined at an ionic strength of $\mu = 0.05$ (Fig. 5a and b). The DAA-70–0 adsorbent satisfactorily removed endotoxin from a BSA solution without affecting the recovery of BSA over a wide pH range from 5 to 8, as shown in Fig. 5a. In contrast, the immobilized histidine had a high endotoxin-removing activity over a wide pH range from 5 to 8, but

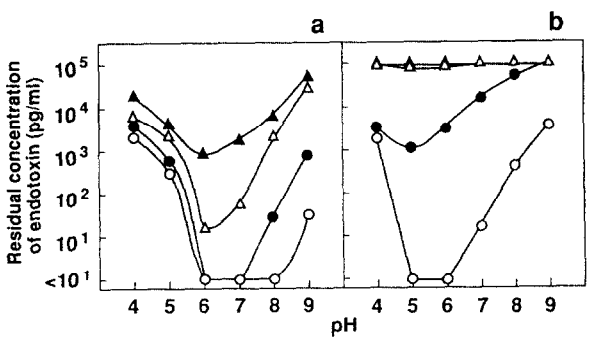

Fig. 4. Effects of pH and ionic strength on removal of endotoxin by (a) DAA-70–0 adsorbent and (b) immobilized histidine. The removal of endotoxin was determined by the batchwise method with 0.3 g of the wet adsorbent and 3 ml of an endotoxin solution (*E. coli* O111:B4 LPS, 100 ng/ml, pH 4–9, $\mu = 0.05$–0.4). Ionic strength: μ = (○) 0.05, (●) 0.1, (△) 0.2 and (▲) 0.4.

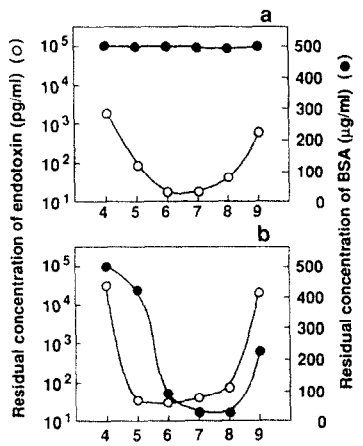

Fig. 5. Effects of pH (x-axes) on removal of endotoxin from a BSA solution containing endotoxin with (a) DAA-70–0 adsorbent and (b) immobilized histidine. The removal of endotoxin was determined by the batchwise method with 0.3 g of wet adsorbent and 3 ml of a BSA solution (endotoxin 100 ng/ml as *E. coli* O111:B4 LPS, BSA 500 μg/ml, pH 4–9, $\mu = 0.05$). ○ = Endotoxin; ● = BSA.

the residual concentration of BSA also decreased from 500 to less than 10 μg/ml, as shown in Fig. 5b. As the ionic strength increased ($\mu = 0.2$) the adsorption of BSA by immobilized histidine decreased (Fig. 2), but this was accompanied by a decrease in the adsorption of endotoxin at any pH value (Fig. 4b). Immobilized histidine therefore cannot remove endotoxin from a BSA solution (500 μg/ml) at any ionic strength or any pH.

On the basis of these results (Figs. 2–5), we assumed that the adsorption of endotoxin and BSA by the adsorbent was induced by cooperation of cationic and weakly hydrophobic properties. The charge of the DAA adsorbent is cationic at pH < 10 because of the amino groups originating from the DAA copolymer. The adsorption of endotoxin was dependent on pH and ionic strength, as shown in Figs. 4 and 5. This suggests that the ionic interaction participates in binding between the adsorbent and endotoxins. The adsorption, being independent of the ionic strength, suggests a hydrophobic interaction; the DAA adsorbent can retain the high endotoxin-removing activity even at a high ionic strength of $\mu = 0.1$–0.4 (Fig. 4a). Immobilized histidine adsorbed endotoxin mainly by ionic interaction at a

low ionic strength, as it adsorbed little endotoxin at high ionic strengths, $\mu = 0.2$–0.4 (Fig. 4b). Presumably, the high endotoxin-removing activity of the DAA adsorbent is related to the ionic interaction between the cationic region of the adsorbents and the anionic region (the phosphoric acid group) of endotoxin, and to the weak hydrophobic interaction between the alkyl chain region of the adsorbent and the lipid region of endotoxin.

It seems that the adsorption of BSA by the adsorbent is induced by the cationic properties. The charge of BSA is anionic at pH values greater than its p*I* (4.9), and also the adsorption of BSA by the adsorbents is dependent on the ionic strength, as shown in Fig. 2. This suggests the participation of the ionic interaction in binding between the adsorbent and BSA; however, the DAA-70–0 adsorbent with $M_{lim} < 300$, which is less than the molecular mass of BSA, adsorbed little BSA at any pH value (Fig. 5a). The adsorption of BSA increased with increase in M_{lim} of the DAA adsorbent, at a low ionic strength, as shown in Fig. 2. Immobilized histidine with large pore sizes ($M_{lim} = 100\,000$) adsorbed a considerable amount of BSA at pH values greater than the p*I* (4.9) of BSA (Fig. 5b). These results show that adsorption of BSA is caused by entry of the BSA molecules into the pores of the adsorbent. Much endotoxin, however, was adsorbed by the DAA-70–0 adsorbent. We reported previously [10] that endotoxin cannot enter the pores of the adsorbent with $M_{lim} <$ 300 because endotoxin aggregates to form supermolecular assemblies ($M_{lim} > 1\,000\,000$). Therefore, we assume that endotoxin is adsorbed also on the surface of the DAA-70–0 adsorbent but BSA can hardly be adsorbed on it.

3.2. Removal of endotoxin from various protein solutions

Table 3 shows the selective removal of endotoxin with the DAA-70–0 adsorbent from various useful protein solutions containing natural endotoxin. BSA, myoglobin, γ-globulin and cytochrome *c* were used as test samples at various concentrations. The proteins were naturally contaminated with endotoxin at concentrations from 500 to 32 000 pg/ml. When the endotoxin adsorption was measured at pH 7.0 and $\mu = 0.05$, a high recovery of protein was obtained with each sample solution after removal of endotoxin. It is essential to eliminate endotoxin or at least to decrease it to a concentration of 100 pg/ml from the fluid to be used for intravenous injection [5], because of its potent biological activity eliciting pyrogenic and shock reactions in mammals [1]. As shown in Table 3, the DAA-70–0 adsorbent was able to remove endotoxin from any sample to a level below 100 pg/ml without loss of the proteins.

3.3. Adsorption capacity and regeneration

The endotoxin-adsorbing capacity of DAA-70–0 adsorbent was determined by frontal chromatography. An endotoxin solution (*E. coli* O111:B4 LPS, 1000 ng/ml) was applied to a column (10 × 0.3 cm I.D.) of the DAA-70–0

Table 3
Removal of endotoxin from various protein solutions with DAA-70–0 adsorbent

Sample (500 μg/ml)	Concentration of endotoxin (pg/ml)		Endotoxin removed (%)	Recovery of protein (%)
	Before treatment	After treatment		
BSA	32 000	80	99	99
Myoglobin	500	20	96	99
γ-Globulin	8400	50	99	99
Cytochrome *c*	800	20	98	100

The endotoxin removed was determined by the batchwise method with 0.5 ml of wet DAA-70–0 adsorbent ($M_{lim} < 300$, amino-group content 4.5 mequiv./g) and 5 ml of a sample solution (pH 7.0, $\mu = 0.05$).

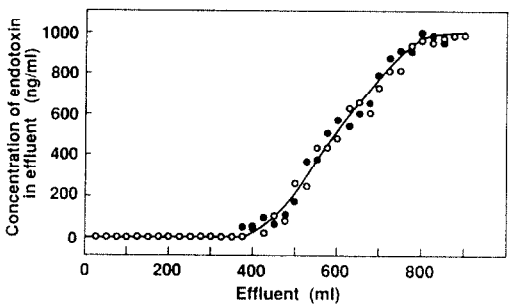

Fig. 6. Adsorption capacity of DAA-70–0 adsorbent for endotoxin (*E. coli* O111:B4 LPS, 1000 ng/ml, pH 7.0, μ = 0.05) in frontal chromatography on a 10 × 0.3 cm I.D. glass column. ○ = First use; ● = fifth use (reused after treatment with 20 ml of 0.2 *M* sodium hydroxide solution followed by 20 ml of 2 *M* sodium chloride solution).

adsorbent. As shown in Fig. 6, the adsorbent was gradually saturated with endotoxin. When the endotoxin-saturated column was washed with 20 ml of a 0.2 *M* sodium hydroxide solution followed by 20 ml of 2 *M* sodium chloride solution, the graph of the adsorbing capacity of the regenerated column was similar to that of the fresh column. We also repeated the column reuse and the 0.2 *M* NaOH treatment five times. As shown in Fig. 6, the curve of the adsorbing capacity of the five-times regenerated column was similar to that of the fresh column. These results show that the DAA adsorbent can be completely regenerated by the 0.2 *M* NaOH treatment. The adsorption capacity of 1 ml of the DAA-70–0 adsorbent was 0.87 mg of endotoxin (*E. coli* O111:B4 LPS) at pH 7.0 and at an ionic strength of $\mu = 0.05$ in the column method. Minobe *et al.* [5] found that the endotoxin-adsorbing capacity of 1 ml of immobilized histidine was 0.53 mg (*E. coli* O128:B12 LPS) at an ionic strength of $\mu = 0.02$. We also found that the adsorbing capacity of 1 ml of the aminated PMLG adsorbent ($M_{lim} = 20\,000$, amino-group content 3.2 mequiv./g) was over 2 mg of endotoxin (*E. coli* O111:B4 LPS) even at a high ionic strength, $\mu = 0.17$, as reported previously [9]. Although the adsorption capacity is lower than that of the aminated PMLG adsorbent, the DAA-70–0 adsorbent can satisfactorily remove endotoxin from a protein solution naturally contaminated with endotoxin, as shown in Table 3.

For practical application, the ease of regeneration is important, and the DAA-70–0 adsorbent can easily be reused, as shown in Fig. 6. The aminated PMLG adsorbent can also be reused after washing by the same method as used for the DAA-70–0 adsorbent. We suspect, however, that the aminated PMLG adsorbent will be gradually hydrolysed in a 0.2 *M* sodium hydroxide solution (one of the solvents used for washing) because the adsorbent has free –CO–O– bonds originating from PMLG. In contrast, the DAA adsorbent, being composed of –CONH– bonds from DMAPAA and AAA, is considered to keep its entire structure in 0.2 *M* sodium hydroxide solution. It seems that this favourable property permits the DAA column to be reused many times.

4. Conclusion

The present results indicate that adsorption technique using the DAA adsorbent can remove endotoxin from a naturally contaminated protein solution without affecting the recovery of the protein. In addition, the DAA adsorbents have the following advantages: (1) they are prepared by one-step polymerization; (2) the amino-group content of the adsorbent is easily adjusted by changing the molar ratio of the monomer; and (3) they can be completely regenerated by washing with several kinds of solutions.

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