Partially-Hydrophobized Polymer Particles Derived from *N,N*-Dimethylaminopropylacrylamide for Endotoxin Removal from Acidic Protein Solution

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Novel copolymeric adsorbents for the selective removal of endotoxin from an acidic protein solution were prepared. The adsorbents comprise spherical copolymers derived from *N,N*-dimethylaminopropylacrylamide (DMAPAA) and divinylbenzene (DVB). When the molar ratio of DMAPAA to DVB was 80/20 (amino-group content: 5.1 meq/g) and the pore size (molecular mass exclusion of polysaccharide, M_{lim}) was 4000 to 10000, DMAPAA/DVB showed high endotoxin-adsorbing activity at pH 5.0 to 9.0 and ionic strengths of $\mu=0.05$ to 0.4. The capacity of the adsorbent (M_{lim}: 4000) was 390 μg of endotoxin (lipopolysaccharide purified from *E. coli* O111 : B4) per ml of the adsorbent using the batchwise method. The apparent dissociation constant between endotoxin and the adsorbent was 2.2×10^{-12} M. On the other hand, the adsorption of bovine serum albumin, an acidic protein, by the adsorbent increased with an increase in M_{lim} from 4000 to 10000, but decreased with an increase in ionic strength (μ) from 0.05 to 0.2. As a result, DMAPAA/DVB (80/20) (M_{lim}: 4000) selectively removed endotoxin from various acidic protein solutions at pH 7.0 and $\mu=0.05$. The residual concentration of endotoxin in the protein solution always decreased to a concentration lower than 0.1 ng/ml, and recovery of the protein was more than 97%.

Key words endotoxin; lipopolysaccharide; *N,N*-dimethylaminopropylacrylamide; divinylbenzene; bovine serum albumin

The removal of endotoxin (lipopolysaccharide: LPS) from substances used as drugs is very important, since LPS causes pyrogenic and shock reactions in mammals upon intravenous injection, even in nanogram quantities.[1-3] Endotoxins, constituents of the cell wall of gram-negative bacteria, are potential contaminants of physiological fluids and aqueous solutions and are very stable, resisting extreme temperatures and pH values. To remove endotoxin from solutions of high-molecular-weight compounds, such as proteins, the adsorption method has proved to be most effective.

We previously reported that aminated poly(γ-methyl L-glutamate) (PMLG–NH_2) spherical particles can selectively remove endotoxin from various protein solutions at a high ionic strength, $\mu=0.2$ to 0.8.[4-7] However, the adsorbing capacity of PMLG–NH_2 was high for both endotoxins and acidic protein, such as bovine serum albumin (BSA), at as low an ionic strength as $\mu=0.05$.[6] We reported also that spherical copolymers of *N,N*-dimethylaminopropylacrylamide (DMAPAA) and *N*-allylacrylamide (AA) could selectively adsorb endotoxin from a BSA solution even at a low ionic strength.[8] However, the endotoxin-adsorbing activity of DMAPAA/AA was not satisfactorily high.

In this study, we developed novel spherical copolymers of DMAPAA and divinylbenzene (DVB). DVB is a more hydrophobic cross-linking agent than AA. To increase its endotoxin-adsorbing activity, it was necessary to increase not only the cationic property but also the hydrophobic property of the adsorbent, because endotoxin is an amphipathic substance having both anionic and hydrophobic regions.[9] This report describes the synthesis of DMAPAA/DVB adsorbents by one-step polymerization and the effects of ionic strength, pH, and pore size on the selective removal of endotoxin from various acidic protein solutions by DMAPAA/DVB and DMAPAA/AA spherical copolymers.

Experimental

Materials Purified endotoxin (*Escherichia coli* UKT-B) and Limulus ES-J test Wako (Limulus amoebocyte lysate) were purchased from Wako Pure Chemical Ind., Ltd., Osaka. Other endotoxins were purchased from Difco Laboratories, Detroit, U.S.A. DMAPAA [$CH_2=CHCONH-(CH_2)_3\cdot N(CH_3)_2$] monomer (Kohjin Co., Ltd., Tokyo) and AA [$CH_2=CHCONHCH_2CH=CH)_2$] monomer (Kohjin) were purified by vacuum distillation at 131 °C (1 mmHg), 115 °C (0.7 mmHg). DVB [$C_6H_4(CH=CH_2)_2$] (Wako) was purified by extraction with 0.1 M sodium hydroxide. Proteins were purchased from Nacalai Tesque, Kyoto. Endotoxin-free water was kindly supplied by Chemo-Sero-Therapeutic Research Institute, Kumamoto. All other chemicals were of analytical reagent grade.

Preparation of DMAPAA/DVB Adsorbents DMAPAA, DVB, diethylbenzene as a diluent, and 2 wt% azobisisobutyronitrile as an initiator were mixed at room temperature. The mixture was added to a 25 wt% anhydrous sodium sulfate solution containing 1% sodium carboxymethyl cellulose. The suspension was stirred and heated at 80 °C for 12 h. The DMAPAA/DVB copolymer particles obtained were washed successively with cold and hot water, methanol and ethanol. Particles with diameters of 44 to 105 μm were used as adsorbents.

Preparation of Other Adsorbents DMAPAA/AA copolymer particles were prepared by one-step polymerization as reported before.[8] Particles with diameters of 44 to 105 μm, amino-group contents of 5.1 meq/g and a pore size of 4000 as molecular mass exclusion (M_{lim}) were used as the adsorbent.

PMLG–NH_2 spherical particles were prepared by the suspension-evaporation method[10,11] and aminolysis[4] with diaminoethane, as described previously. Particles with diameters of 44 to 105 μm, amino-group contents of 3.5 meq/g and M_{lim} of 8000 were used as the adsorbent.

Determination of Amino Group Contents of Adsorbents The amino groups were quantified by pH titration and elemental analysis as previously described.[4]

Determination of Pore Size and Hydrophobicity of the Adsorbent The adsorbents prepared were each packed into a stainless column (4.6 × 100 mm). The chromatograph was equipped with a JASCO 880-PU pump and a Shodex refractomonitor SE-51. The pore size (M_{lim}) of the matrix in the adsorbent was estimated from calibration curves by aqueous size exclusion chromatography (SEC) as described previously.[10-12] Pullulan standards were used as permeable substances. The hydrophobicity of the

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adsorbent was estimated from the elution behavior of 1-alcohol obtained by SEC with the adsorbent-packed column. 1-Methanol, 1-ethanol, 1-propanol and 1-butanol were used as permeable substances.

Adsorption of Endotoxin and Other Substances Endotoxin was dissolved in the following buffers: 0.02 M sodium acetate (pH 4, 5); 0.02 M phosphate (pH 6, 7, 8); and 0.02 M Tris (pH 9, 10). The ionic strength of the buffer was adjusted by changing the sodium chloride concentration. The adsorption of endotoxin was measured by a batchwise method as follows: The adsorbent was washed and equilibrated with one of the various buffers with different ionic strengths. A 0.1- to 0.2-g portion of wet adsorbent was suspended in 2 to 4 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 filtrate was measured for endotoxin content. The apparent dissociation constant ($K_{d,app}$) between endotoxin and adsorbent was estimated by adsorption isotherm, as described previously.[8,13,14] The adsorption of other substances was estimated by the same method as endotoxin-binding assay. The adsorbing activities for endotoxin and other substances were shown by an average of five measurments.

Endotoxin Assay Endotoxin was assayed by the Limulus test involving a turbidimetric time assay at 660 nm with a Toxinometer ET-201 (Wako).[15] Purified endotoxin (*E. coli* UKT-B) was used as the standard. Limulus ES-J test Wako was used as the reagent for the reaction.

Protein Assay Protein concentration was measured at 280 nm with a Spectrophotometer UV-160 (Shimadzu).

Results and Discussion

Preparation of Adsorbents We reported previously that DMAPAA/AA with an amino-group content of over 4.5 meq/g showed high endotoxin-adsorbing capacity, and that the adsorbents with M_{lim} of over 10000 (the molecular weight of polysaccharide) adsorbed BSA.[8] To achieve selective adsorption of endotoxin, it was necessary to reduce the interaction of the adsorbent with protein. Therefore, as shown in Table 1, DMAPAA/DVB adsorbents with amino-group contents of 4.0 to 5.1 meq/g and pore sizes of 4000 to 10000 as M_{lim} were prepared. The amino-group content and M_{lim} were easily adjusted by changing the ratio of DMAPAA and the ratio of diluent in polymerization. The amino-group content increased from 4.0 to 5.1 meq/g with an increase in the ratio of DMAPAA from 60 to 80 mol%, and M_{lim} increased from 4000 to 10000 with an increase in the diluent ratio (to the DMAPAA/DVB solution) from 20 to 100 vol%. In order to compare the endotoxin-adsorbing activity of DMAPAA/DVB, DMAPAA/AA (AA-20-20) (amino-group content: 5.1 meq/g, M_{lim}: 4000) was prepared by one-step polymerization as reported before.

Figure 1 shows the elution behaviors of 1-alcohols by SEC with the columns packed with each of DMAPAA/DVB (DVB-30-20, -20-20) and DMAPAA/AA (AA-20-20). The hydrophobicity of 1-alcohol increases with an increase in its carbon number from 1 (methanol) to 4 (butanol).[15] The elution volume of 1-alcohol increases with an increase in the hydrophobicity of the adsorbent, and the difference in the elution volume between 1-methanol and 1-butanol ($V_{BuOH-MeOH}$) also increases. $V_{BuOH-MeOH}$ of the adsorbent increased from 13 to 116 vol% with an increase in the DVB ratio from 0 (AA-20-20) to 30 (DVB-30-20) mol%. This result indicates that the adsorbent became hydrophobic with the introduction of DVB.

Effects of Various Factors on Selective Adsorption of Endotoxin The endotoxin-adsorbing activities of the adsorbents were examined by the batchwise method with various kinds of buffers. Standard LPS from *E. coli* O111 : B4 was used as an endotoxin-containing sample.

The effects of ionic strength on endotoxin adsorption were examined with DMAPAA/DVB (amino-group content: 4.0 to 5.1 meq/g, M_{lim}: 4000) and DMAPAA/AA (amino-group content: 5.1 meq/g, M_{lim}: 4000). As shown in Fig. 2, the higher the ionic strength of the buffer, the lower the endotoxin-adsorbing activity of all adsorbents. At $\mu=0.05$ to 0.2 and pH 7.0, DMAPAA/DVB with amino-group contents of 4.5 (DVB-30-20) or 5.1 meq/g (DVB-20-20) showed a high endotoxin-adsorbing activity. DMAPAA/AA (AA-20-20) showed high adsorption of endotoxin only at $\mu=0.05$ and pH 7.0. DMAPAA/DVB with M_{lim} of 8000 (DVB-20-60) and 10000 (DVB-20-100) also showed high endotoxin-adsorbing activities, similar to that of DVB-20-20.

The effects of pH and ionic strength on endotoxin adsorption by DVB-20-20 and AA-20-20 are shown in Fig. 3. When the ionic strength of the buffer was adjusted to $\mu=0.05$, each adsorbent adsorbed endotoxin satisfactorily at around neutral pH. When the ionic strength was increased to $\mu=0.2$ or higher, the endotoxin-adsorbing activity of AA-20-20 decreased markedly at any pH value (Fig. 3b). Even at high ionic strength values ($\mu=0.2$, 0.4), DVB-20-20 showed a high endotoxin-adsorbing activity at pH 5 to 9 (Fig. 3a).

The adsorption isotherms for DVB-20-20 and AA-20-20 were determined by the batchwise method in a phosphate buffer (pH 7.0, $\mu=0.05$) by changing the concentration of endotoxin (*E. coli* O111 : B4 LPS). The endotoxin adsorption by each adsorbent increased with an increase in the concentration of endotoxin (Fig. 4a). Figure 4b and

Table 1. Properties of Cross-Linked DMAPAA Adsorbents

Adsorbent	Molar ratio (mol %)			Diluent (vol %)	M_{lim}[c]	Amino-group content[d] (meq/g)	Sd[e] (wet-ml/dry-g)
	DMAPAA	DVB	AA				
DVB-40-20	60	40	0	20[a]	4000	4.0	3.5
DVB-30-20	70	30	0	20[a]	4000	4.5	3.9
DVB-20-20	80	20	0	20[a]	4000	5.1	4.0
DVB-20-60	80	20	0	60[a]	8000	5.1	6.2
DVB-20-100	80	20	0	100[a]	10000	5.1	6.5
AA-20-20	80	0	20	20[b]	4000	5.1	5.0

a) Vol % of diethylbenzene to DMAPAA–DVB solution. *b*) Vol % 1-hexanol to DMAPAA–AA solution. *c*) Value deduced as a molecular weight of polysaccharide. *d*) Amino groups introduced into the adsorbent. *e*) Degree of swelling in water.

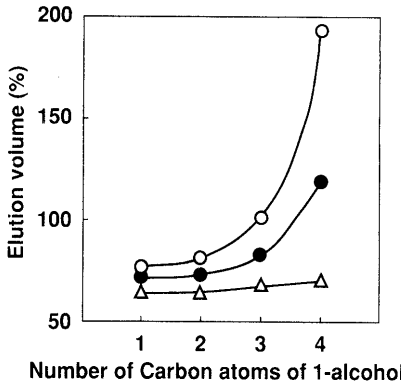

Fig. 1. Elution Behaviors of 1-Alcohols with Various Cross-Linked DMAPAA Columns

A 5-μl portion of 1-alcohol dissolved in water (2 mg/l) was injected to the column (100 × 4.6 mm i.d.) at 0.5 ml/min and at 25 °C. The elution volume of 1-alcohol is expressed as a percentage of the column volume. Column and DVB contents: ○ = DVB-30-20, 30 mol%; ● = DVB-20-20, 20 mol%; △ = AA-20-20, 0 mol%. Number of carbon atoms of 1-alcohol: 1 = methanol; 2 = ethanol; 3 = propanol; 4 = butanol. Pore size (M_{lim}) of column: 4000.

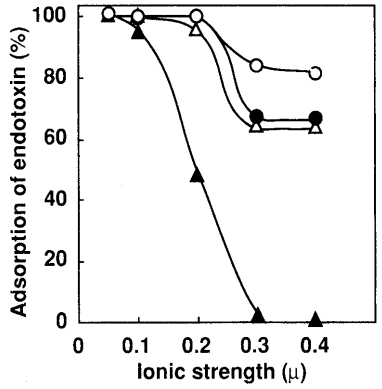

Fig. 2. Effects of Ionic Strength on Adsorption of Endotoxin to DMAPAA/DVB and DMAPAA/AA Adsorbents

The adsorption of endotoxin was determined by the batchwise method with 0.2 g of wet adsorbent and 2 ml of an endotoxin solution (LPS from *E. coli* O111 : B4, 500 ng/ml, pH 7.0, μ = 0.05 to 0.4). Adsorbent and amino-group content: ○ = DVB-20-20, 5.1 meq/g; ● = DVB-30-20, 4.5 meq/g; △ = DVB-40-20, 4.0 meq/g; ▲ = AA-20-20, 5.1 meq/g. M_{lim} of adsorbent: 4000.

c show the Scatchard plots,[13,14] derived from these adsorption isotherms (Fig. 4a). According to these plots, the endotoxin-adsorbing capacities of 1 g of wet adsorbent and the endotoxin-adsorbing constants were calculated at 470 μg (390 μg/ml-adsorbent) and 4.5×10^{11} M^{-1} for DVB-20-20 (Fig. 4b), and 360 μg (240 μg/ml-adsorbent) and 2.5×10^{10} M^{-1} for AA-20-20 (Fig. 4c), when the aggregation weight of endotoxin was estimated at one million.[13] That is, the $K_{d,app}$ values between adsorbent and endotoxin were 2.2×10^{-12} M for DVB-20-20, and 4.0×10^{-11} M for AA-20-20. $K_{d,app}$ of DVB-20-20 was about 1/20 as small as that of AA-20-20, although its amino-group content was similar to that of AA-20-20. These results indicate that the endotoxin-adsorbing activity of DVB-20-20 is higher than that of AA-20-20.

Endotoxin is an amphipathic substance having both anionic (phosphate groups) and hydrophobic regions (lipophilic groups).[9] The adsorption, being dependent on ionic strength and pH values, suggests a cationic interaction. The adsorption, being independent of ionic strength, suggests a hydrophobic interaction. AA-20-20 adsorbs endotoxin mainly by cationic interaction originat-

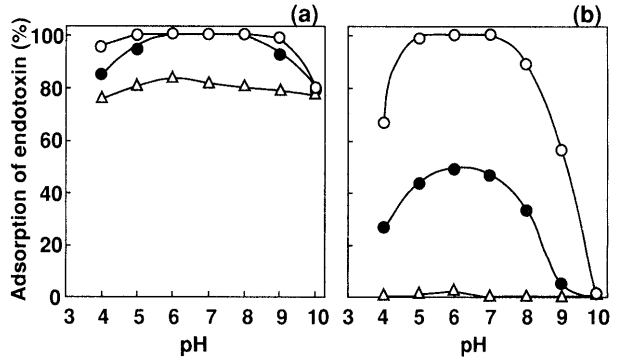

Fig. 3. Effects of pH and Ionic Strength on Adsorption of Endotoxin to DMP/DVB (DVB-20-20) (a) and DMP/AA (AA-20-20) (b)

The adsorption of endotoxin was determined by the batchwise method with 0.2 g of wet adsorbent and 2 ml of an endotoxin solution (LPS from *E. coli* O111 : B4, 500 ng/ml, pH 4.0 to 10.0, μ = 0.05 to 0.4). Ionic strength (μ): ○ = 0.05; ● = 0.2; △ = 0.4. Amino-group content of adsorbent: 5.1 meq/g. M_{lim} of adsorbent: 4000.

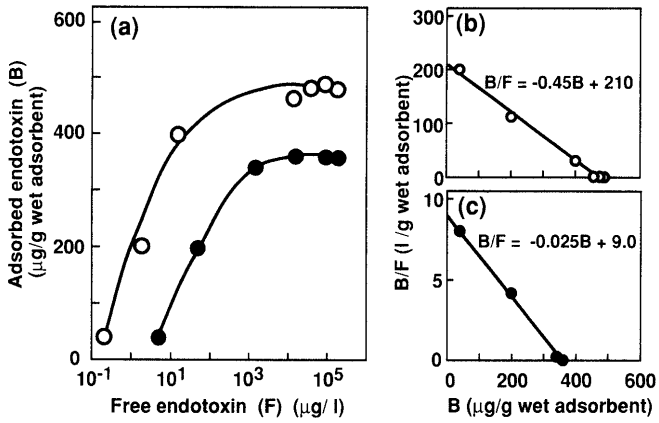

Fig. 4. Endotoxin-Adsorption Isotherms (a) and Scatchard Plots (b, c)

The adsorption isotherms for DVB-20-20 (○) and AA-20-20 (●) were determined by the batchwise method with 0.1 g of the wet adsorbent and 4 ml of an endotoxin solution (LPS from *E. coli* O111 : B4, 10^3 to 2×10^5 ng/ml, pH 7.0, μ = 0.05). Scatchard plots[13,14] were derived from (a). Scatchard plots: (b) = DVB-20-20; (c) = AA-20-20.

ing from DMAPAA, because it adsorbs less endotoxin at such high ionic strength values: at μ = 0.2—0.4, pH 7.0 (Figs. 2, 3). By contrast, DVB-20-20 can retain about 80% of the endotoxin-adsorbing rate even at a high ionic strength of μ = 0.4 (Fig. 2). This result suggests that both the cationic property originating from DMAPAA and the hydrophobic property originating from DVB markedly increase the endotoxin-adsorbing activity of DVB-20-20.

For the selective adsorption of endotoxin, it is necessary to reduce the interaction between the adsorbent and protein. The effects of M_{lim} and the ionic strength of the adsorbent on the adsorption of BSA, an acidic protein, were examined with various adsorbents at μ = 0.05 to 0.4. DMAPAA/DVB adsorbents with an amino-group content of 5.1 meq/g and M_{lim} of 4000 to 10000 (DVB-20-10, 20-50, 20-100) were used (Fig. 5). The BSA-adsorption rate of DVB-20-10 (M_{lim}: 4000) was <2% at any ionic strength. The BSA-adsorbing capacities of other adsorbents increased with a decrease in ionic strength. At a low ionic strength (μ = 0.05), the adsorption rate of BSA increased from <2% to 83% with M_{lim} of the adsorbent from 4000 to 10000. DMAPAA/AA with a large pore size (M_{lim} over 10000) also showed high BSA-adsorbing activity at such a low ionic strength as μ = 0.05.[8] These

results suggest that DMAPAA/DVB with M_{lim} of 4000 or below can adsorb endotoxin without affecting the BSA recovery.

The adsorption of endotoxins originating from various gram-negative bacteria to DVB-20-20 was compared with that to AA-20-20 (Table 2). At pH 7.0 and $\mu=0.05$ or 0.2, the adsorption of various endotoxins to DVB-20-20 was higher than to AA-20-20. DVB-20-20 lowered the concentration of any endotoxin from 500 to 0.1 ng/ml or lower at $\mu=0.05$ and pH 7.0.

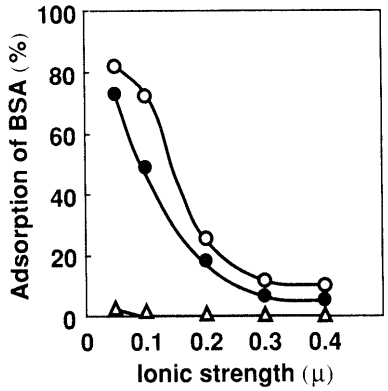

Fig. 5. Effects of Pore Size and Ionic Strength on Adsorption of BSA to DMAPAA/DVB

The BSA adsorption was determined by the batchwise method with 0.2 g of wet adsorbent and 2 ml of BSA solution (500 μg/ml, pH 7.0, $\mu=0.05$ to 0.4). M_{lim}: ○=10000; ●=8000; △=4000. Amino-group content: 5.1 meq/g.

Table 2. Adsorption of Endotoxin by DMAPPAA/DVB and DMAPAA–AA

Endotoxin[a]	Residual concentration of endotoxin after treatmemt[b] (ng/ml)			
	DVB-20-20 adsorbent		AA-20-20 adsorbent	
	$\mu=0.05$	$\mu=0.2$	$\mu=0.05$	$\mu=0.2$
Escherichia coli UKT-B	<0.01	<0.01	<0.01	280
Escherichia coli O111:B4	<0.01	<0.01	<0.01	200
Escherichia coli O55:B5	0.02	0.04	0.3	380
Escherichia coli O127:B8	0.05	5.0	2.0	450
Salmonella typhimurium	0.02	2.4	4.0	420
Salmonella typhosa O901	<0.02	0.3	0.7	270
Bordetella pertussis TOHAMA	<0.01	<0.01	0.08	350

a) The purified endotoxin was dissolved in phosphate buffer (pH 7.0, $\mu=0.05$, 0.2) 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.

The effects of the ionic strength of buffers on the selective removal of endotoxin from a BSA solution with various adsorbents were examined. As shown in Fig. 6a, DVB-20-20 (amino-group content: 5.1 meq/g, M_{lim}: 4000) selectively removed endotoxin from a BSA solution at $\mu=0.05$ to 0.2. AA-20-20 (amino-group content: 5.1 meq/g, M_{lim}: 4000) adsorbed endotoxin only at a low ionic strength of $\mu=0.05$ (Fig. 6b). The adsorbent was unsatisfactory with respect to endotoxin-adsorbing activity; the concentration of endotoxin remaining after treatment was 18 ng/ml at $\mu=0.05$ and pH 7.0. Although PMLG–NH_2 (amino-group content: 3.5 meq/g, M_{lim}: 8000) showed high endotoxin-adsorbing activities for both endotoxin and BSA at $\mu=0.05$, they selectively removed endotoxin at $\mu=0.2$ to 0.4 (Fig. 6c). We previously reported that PMLG–NH_2 maintained a high endotoxin-removing activity even at such a high ionic strength as $\mu=1.0$,[5-7] probably because the hydrophobicity[16] of PMLG is higher than those of DMAPAA/AA and DMAPAA/DVB.

The charge of protein is anionic at pH over pI and cationic at pH under it. Accordingly, the most favorable pH for the selective removal of endotoxin from a protein solution is below its pI and above the pK_a of the phosphate residues of endotoxin. Most adsorbents we used readily removed endotoxin from cytochrome c (a basic protein) and γ-globulin (a neutral protein) solutions at pH 7 without a loss of protein.[6,8,14] This is because the ionic interaction of the adsorbent with cytochrome c (pI 10.6) and γ-globulin (pI 7.4) is not induced at pH 7.0 (lower than pI).

In contrast, high-molecular weight acidic substances such as BSA are anionic at pH 7.0 (over pI), and are adsorbed considerably by cationic adsorbents. To selectively adsorb endotoxin in an BSA solution, it is necessary to decrease the pH value of the buffer to 4.9 (pI of BSA) or lower, but at such a low pH, the endotoxin-adsorbing activity of the adsorbent decreases (Fig. 3).

On the other hand, the adsorption of BSA is dependent on the pore size of the matrix. As shown in Figs. 5 and 6, the adsorbents with M_{lim} over 4000 (DVB-20-50, -20-100, and PMLG–NH_2 adsorbent) adsorbed BSA at $\mu=0.05$ and pH 7. Little BSA was adsorbed by DVB-

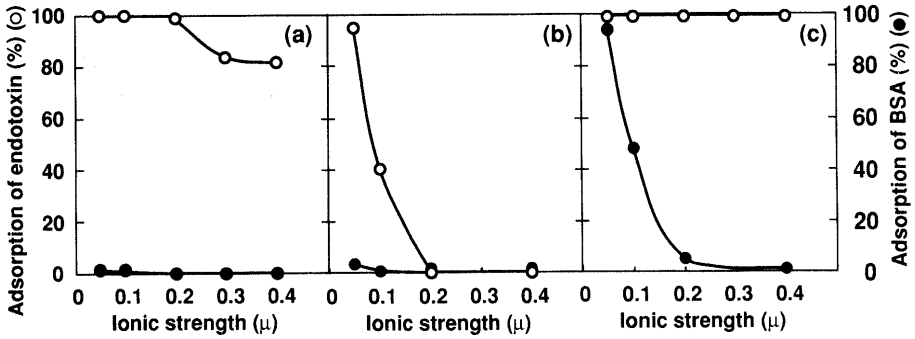

Fig. 6. Effects of Ionic Strength on the Removal of Endotoxins from a BSA Solution by Various Adsorbents

The removal of endotoxin was determined by the batchwise method with 0.2 g of wet adsorbent and 2 ml of sample solution (BSA, 500 μg/ml; LPS from *E. coli* O111:B4, 500 ng/ml; pH 7.0; $\mu=0.05$ to 0.4). Adsorbent, M_{lim}, and amino-group content (meq/g): (a)=DVB-20-20, 4000, 5.1; (b) AA-20-20, 4000, 5.1; (c) aminated PMLG-NH_2, 8000, 3.5.

Table 3. Removal of Endotoxin from Various Protein Solutions with DMAPAA/DVB and DMAPAA/AA

Sample solution			DVB-20-20 adsorbent		AA-20-20 adsorbent	
Protein	pI	Endotoxin conc.[a] before treatment (ng/ml)	Endotoxin conc. after treatment (ng/ml)	Recovery of protein (%)	Endotoxin conc. after treatment (ng/ml)	Recovery of protein (%)
Egg albumin	4.6	32	0.07	97	8.1	98
BSA	4.9	80	0.08	98	6.4	99
β-Lactoglobulin	5.1	25	0.05	97	2.5	98
Insulin	5.8	53	0.03	99	5.4	99

a) Endotoxin naturally contaminating protein solution. Endotoxin removal was determined by the batchwise method with 0.2 g of wet adsorbent and 2 ml of a protein solution (1 mg/ml, pH 7.0, $\mu=0.05$) containing endotoxin.

20-20 or DAA-20-20 (M_{lim}: 4000) at any ionic strength. We previously found that histidine-immobilized agarose spheres with a large pore size (M_{lim}: 100000), a commercially available adsorbent, had high endotoxin-adsorbing activity at $\mu=0.05$ to 0.1 and at neutral pH, but BSA was also adsorbed efficiently.[8,13,14] These results show that the adsorption of BSA is caused by its entry into the pores of the adsorbent.

We previously reported that endotoxin cannot enter such pores with $M_{lim}<400000$, because it forms a supermolecular aggregate ($M_{lim}>1000000$).[6] Endotoxin, however, is adsorbed very efficiently by DMAPAA/DVB (M_{lim}: 4000). Therefore, we assume that endotoxin is adsorbed also to the surface of DMAPAA/DVB, but BSA is not.

It is suggested that the DMAPAA/DVB adsorbents bind endotoxin more strongly than BSA, even at a low ionic strength, probably because the adsorbent can adsorb endotoxin not only through their cationic properties but also through their hydrophobic properties, and because endotoxins have lower pK_a and higher hydrophobicity than BSA.

These results (Figs. 4, 5, 6) show that it is effective for the selective adsorption of endotoxin to adjust the amino-group content of the adsorbent to 5.1 meq/g and the pore size (M_{lim}) to 4000 or lower. However, it is difficult to adjust M_{lim} of the adsorbent when the ligand is introduced to the matrix prepared, because the introduction of ligands such as amino groups often increase the swelling degree of the adsorbent. For example, we observed that the M_{lim} increased from <500 to 8000 by introducing 3.5 meq/g of amino groups in amination of PMLG spheres. In contrast, DMAPAA/DVB and DMAPAA/AA copolymers were prepared by one-step polymerization without amination; therefore it was easy to ajust M_{lim}.

Removal of Endotoxin from Acidic Protein The endotoxin-removing activity of DVB-20-20 was compared with that of AA-20-20 (Table 3). Acidic protein solutions naturally contaminated with endotoxin at concentrations from 25 to 80 ng/ml were used as samples. Each adsorbent showed high recovery (97 to 99%) of protein as well as the efficient removal of endotoxin at pH 7.0 and $\mu=0.05$. It is essential to eliminate endotoxin to at least a concentration lower than 0.1 ng/ml from fluids used for intravenous injection,[13] since it elicits pyrogenic and shock reactions in mammals.[1,2] AA-20-20, however, failed to decrease endotoxin in a protein solution to 0.1 ng/ml. In contrast, DVB-20-20 decreased endotoxin in all samples to lower than 0.1 ng/ml without any loss of protein.

For practical application, the ease of regeneration is important, and we reported previously that DMAPAA/AA can be completely regenerated by washing with 0.2 M sodium hydroxide followed by 2.0 M sodium chloride.[8] DMAPAA/DVB could also be regenerated by the same method as that for DMAPAA/AA (data not shown).

Conclusion

The results suggest that DMAPAA/DVB can remove endotoxin from drugs and fluids for injection to a concentration of 0.1 ng/ml or lower, even at such a low ionic strength as $\mu=0.05$. This process did not affect the recovery of important compounds such as protein. This selective removal activity of DMAPAA/DVB is due to (1) the simultaneous effects of cationic properties of DMAPAA and hydrophobic properties of DVB, and (2) the exempting effects on protein molecules when the M_{lim} rate is ajusted to 4000.

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