Journal of Chromatography, 530 (1990) 148–153
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5352

Note

Removal of endotoxins using macroporous spherical poly(γ-methyl L-glutamate) beads and their derivatives

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(First received January 26th, 1990; revised manuscript received April 9th, 1990)

Removal of endotoxins from substances used as drugs is very important. Recently, the use of affinity chromatography for endotoxin removal, as opposed to conventional physical adsorption and membrane filtration, has attracted special interest. However, although histidine-immobilized polysaccharides [1,2] and chitosan beads [3] are presently sold as endotoxin adsorbents, these are unsatisfactory with respect to selectivity, adsorption capacity and compatibility.

We have developed new adsorbents for the selective removal of endotoxins. The adsorbents are composed of macroporous poly(γ-methyl L-glutamate) (PMLG) spherical beads [4] and their aminated derivatives. Some of the adsorbents can selectively remove 99–100% of the endotoxins in some substances, *e.g.* control standard endotoxin solution from *Escherichia coli* and concentrated toxoids from crude tetanus and diphtheria vaccine materials.

This paper describes new adsorbents for selective removal of endotoxins. The chemical structures of the adsorbents prepared for this study are given in Table I.

EXPERIMENTAL

Materials

Porous A beads with average diameters of 44–105 μm were prepared from PMLG by the "suspension and evaporation" method previously reported [4]. The exclusion molecular mass of the beads was controlled so as to be more than $2 \cdot 10^5$ of the molecular mass of dextran by using di-(2-ethylhexyl)phthalate as a macroreticulating agent [5] in the spherical process. Porous B and C beads were prepared from poly (γ-ethyl L-glutamate) (PELG) and poly(γ-benzyl L-glutamate) (PBLG), respectively. Primary amino groups were introduced into the PMLG beads by aminolysis reaction with various diaminoalkanes (diaminoethane, diaminopropane and diaminobutane) to obtain D, E and F beads, respectively. Methanol, ethanol and decahydronaphthalene–chloroform mixtures proved to

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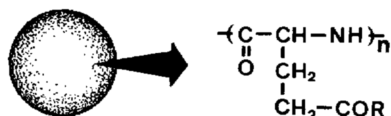
TABLE I

CHEMICAL STRUCTURES OF ADSORBENTS



Adsorbent		Residual group (-R)	Content of residual groups (%)
Number	Abbreviation		
A	PMLG	$-OCH_3$	100
B	PELG	$-OC_2H_5$	100
C	PBLG	$-OCH_2C_6H_5$	100
D-1	PMLG-ED-9	$-NH-(CH_2)_2-NH_2$	9
D-2	PMLG-ED-18	$-NH-(CH_2)_2-NH_2$	18
D-3	PMLG-ED-34	$-NH-(CH_2)_2-NH_2$	34
D-4	PMLG-ED-48	$-NH-(CH_2)_2-NH_2$	48
E	PMLG-PD-26	$-NH-(CH_2)_3-NH_2$	26
F	PMLG-BD-19	$-NH-(CH_2)_4-NH_2$	19

be good media for this reaction. The amount of primary amino group introduced into the beads was adjusted by controlling the reaction conditions, *e.g.* temperature, time, polarity of medium and the ratio of diaminoalkane to the PMLG beads. The amount of amino groups in the beads was determined by pH titration and elemental analysis.

Pyrosep and chitosan beads were purchased from Daicel (Tokyo, Japan) and Kurita (Tokyo, Japan), respectively.

Control standard endotoxins from *E. coli* UKT-B and 0111B4 were purchased from Wako (Osaka, Japan) and Green (Osaka, Japan), respectively. Concentrated toxoids from crude tetanus and diphtheria vaccine materials were obtained from the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan).

Measurements

The affinity of adsorbents for endotoxins was measured by a batchwise method as follows. Adsorbents and endotoxins were dispersed separately in buffer solutions with same concentration and ionic strength. The two solutions were then mixed together. The mixture was shaken for 30 min at 25°C, then the adsorbent was removed by filtration. The assay of endotoxins of the filtrate was carried out using Toxinometer ET-201 (Wako). The adsorption capacity for endotoxins was determined by a frontal column method (5 x 0.4 cm I.D. glass column).

TABLE II

REMOVAL OF ENDOTOXINS FROM VARIOUS SAMPLES

Adsorbent	$-NH_2$ (mequiv./g)	Removal of endotoxin[a] (%)			
		Control standard		Crude toxoid	
		E. coli 0111B4 (250 ng/ml[b], $\mu = 0.17$)	*E. coli* UKT-B (200 ng/ml[b], $\mu = 0.03$)	Tetanus (24 000 ng/ml[b], $\mu = 0.17$)	Diphtheria (3600 ng/ml[b], $\mu = 0.17$)
PMLG	<0.01	78.4 (54)	62.5 (76)	93.0 (1680)	68.1 (1150)
PELG	<0.01	99.6 (1.0)	88.1 (24)		
PBLG	<0.01	100 (<0.01)	86.7 (26)		
PMLG-ED-34	2.27	99.1 (2.3)	100 (<0.01)		
PMLG-ED-48	3.10	99.6 (1.0)	99.3 (3.4)	97.3 (720)	99.9 (3.6)
PMLG-PD-26	1.75	95.5 (11)	99.7 (0.60)	99.4 (144)	
PMLG-BD-19	1.29	92.4 (19)	99.7 (0.60)	99.7 (552)	
Chitosan	6.2[c]	0 (250)	92.8 (18)	0 (24 000)	71.9 (1010)
Pyrosep	>0.3[d]	86.5 (34)	99.7 (0.60)	46.9 (12 700)	53.7 (1670)

[a]The number shows the percentage of endotoxins removed from the sample by stirring for 30 min with adsorbents, and the number in parentheses shows the amount of remaining endotoxin in the effluent; 50 mg of adsorbents were used for 1 ml of endotoxin solution.

[b]Initial concentration of endotoxins in the sample.

[c]Theoretical value.

[d]The content of imidazolyl groups.

Antigen assay of tetanus vaccine materials was carried out by the limit of flocculation test.

RESULTS AND DISCUSSION

The affinity of adsorbents for endotoxins was examined by a batchwise method. Two kinds of control standard endoxotin from *E. coli* and two kinds of concentrated toxoid from crude vaccine materials were used as endotoxin-containing samples.

As summarized in Table II, PMLG derivatives showed good removal of endotoxins from all samples. In each sample, 90–100% of the endotoxins were removed, independent of the concentration, ionic strength or material of the sample. The removal of endotoxins was strongly dependent on the sample type in the case of Pyrosep and the chitosan beads. The adsorption capacitiy of these adsorbents declined with higher concentration of endotoxins or higher ionic strength. Minobe *et al.* [2] also found that the adsorption capacity of imidazole-immobilized polysaccharides such as Pyrosep for endotoxins declined as the ionic strength in the sample increased. This property is very disadvantageous for selective removal of endotoxins from protein-containing samples, because some proteins are themselves adsorbed at lower ionic strengths.

The effectiveness of PMLG derivatives as endotoxin adsorbents increased when a column chromatography process was used. The adsorption capacity of

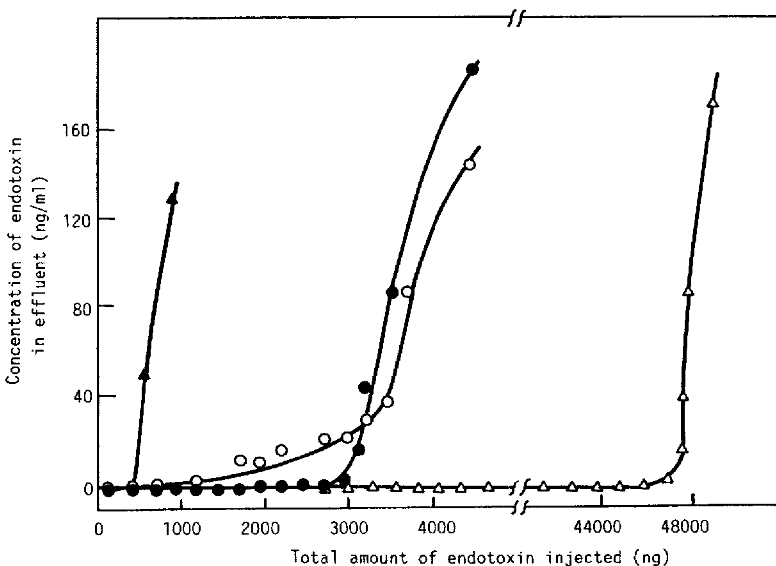


Fig. 1. Adsorption capacity of various adsorbents for endotoxin *E. coli* UKT-B (250 ng/ml, μ = 0.17) in frontal chromatography on a 5.0–8.0 × 0.4 cm I.D. glass column. ○ = PMLG (0.63 bed-ml); ● = PMLG-ED-34 (1.0 bed-ml); △ = PMLG-ED-48 (1.0 bed-ml); ▲ = Pyrosep (1.0 bed-ml).

each adsorbent for endotoxins was determined by frontal chromatography. As shown in Fig. 1, the elution of endotoxin begins with the injection of only 500 ng per 1.0 bed-ml of Pyrosep. This amount increased to 3000 ng per 0.63 bed-ml of PMLG-ED-34 and 48 000 ng per 1.0 bed-ml of PMLG-ED-48. It is clear that the adsorption capacity is much higher in the PMLG derivatives than in Pyrosep. It was also confirmed that the affinity for endotoxins was maintained even in samples with higher ionic strength in the case of the PMLG beads and their derivatives. For example, the adsorption capacities of PMLG and PMLG-ED-17 beads were maintained even when $\mu = 0.3$ and 0.5, respectively.

On the basis of these results, we assumed that the strong affinity of PMLG derivatives for endotoxins was induced by cooperative interactions based on cationic and weak hydrophobic properties of the beads. This assumption was supported as follows. First, from the viewpoint of ionic interaction, (1) the adsorption capacity was increased by the introduction of amino groups into the PMLG beads (Table II), (2) it was further increased as the content of amino groups increased (Fig. 1), and (3) it decreased at very strong ionic strengths, such as $\mu =$ 1.0. Secondly, from the viewpoint of hydrophobic interaction, the non-aminated PMLG beads can adsorb endotoxin regardless of the non-ionic adsorbent (Table II), and the adsorption capacity is higher than that of Pyrosep (Fig. 1). This indicates that the poly(γ-methyl L-glutamate) component has some essential affinity for endotoxins. Although the PMLG beads themselves show a tendency towards hydrophobicity [4,9], the introduction of ethyl or benzyl groups instead of methyl groups increased the hydrophobicity of the adsorbents and led to an increase in the removal of endotoxins (Table II).

Presumably, the good affinity of aminated PMLG derivatives is related to the anionic properties produced from phosphoric acid groups and the hydrophobicity of lipophilic groups of endotoxins [6].

CONCLUSION

This study confirmed that the PMLG beads and their derivatives, especially aminated beads, show a greater adsorption capacity for endotoxins than do con-

TABLE III

REMOVAL OF ENDOTOXIN AND RECOVERY OF ANTIGEN FROM CONCENTRATED TETANUS TOXOID USING PMLG AND PMLG-ED-18 BEADS

Initial concentration of endotoxin in tetanus toxoid was 5980 ng/ml; 200 mg of adsorbents were used for 6 ml of tetanus toxoid.

Adsorbent	Removal of endotoxin (%)	Recovery of antigen (%)
PMLG	98.5	85.7
PMLG-ED-18	99.7	100

ventional adsorbents [1–3,7]. The affinity was maintained even at high ionic strengths. This favourable property enabled the selective removal of endotoxins. As shown in Table III, good recovery of antigen was observed in crude tetanus vaccine materials after removal of endotoxins. In addition, as previously reported [4,8], the PMLG beads show remarkable high flow-rate resistance in liquid chromatography because of their rigid structure of peptide chains and intermolecular hydrogen bonds. Therefore, PMLG beads and their derivatives are quite useful as endotoxin adsorbents in high flow-rate affinity chromatography.

ACKNOWLEDGEMENTS

We thank Mr. K. Aihara and S. Tsunoda of the Chemo-Sero-Therapeutic Research Institute for their technical assistance.

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