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High-performance affinity capture-removal of bacterial pyrogen from solutions

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Abstract

Synthetic peptide S3Δ has high affinity for bacterial endotoxin or lipopolysaccharide (LPS). Under tested conditions of pH 5–9 and 0–0.4 M NaCl, the affinity constant, K_D ranged from $2 \cdot 10^{-6}$ to $2 \cdot 10^{-9} M^{-1}$. A novel affinity matrix based on peptide S3Δ was developed for removal of LPS from solutions such as: water; buffers with a wide range of ionic strength and pH; medium for cell culture; and protein solutions under optimized conditions. At a starting LPS of ≈ 100 EU/ml, a post-purification level below 0.005 EU/ml was achieved. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Endotoxin or lipopolysaccharide (LPS) is an integral component of the outer cell wall of gram-negative bacteria. It causes pyrogenic and endotoxic shock reactions in mammals [1,2]. Development of techniques and materials for decontamination of endotoxin from pharmaceutical and parenteral fluids is therefore crucial for health care products, and has remained a target of much research effort. Various methods have been developed to achieve this aim for different target solutions [3]. These methods involve ultrafiltration for decontamination of water and solutions containing products of low molecular mass,

e.g., glucose, salts, chemotherapeutics, antibiotics and radiopharmaceuticals. Other approaches include two-phase extraction and different kinds of adsorption methods for protein solutions [3]. For removal of LPSs from solutions of cell products, especially proteins, adsorption methods have proven to be the most effective [4]. Different adsorbents have been developed with varying success. These are (a) anion exchangers such as diethylaminoethane (DEAE), (b) affinity matrix using polymyxin B-immobilized Sepharose, histidine-immobilized Sepharose, poly-(ethyleneimine) (PEI), poly-L-lysine (PLL), poly-L-histidine (PLH) and aminated poly(γ -methyl L-glutamate) (PMLG), and (c) immunoaffinity matrix [3,5]. However, many of these adsorbents, particularly, the ion exchangers, are not amenable to operations under wide range of pH and ionic strength. Furthermore, there is a compromise to the efficiency of LPS-

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removal over protein recovery. This problem is exacerbated by poor clearance factor of LPS when the feed concentration is low, which is a common problematic endotoxin contamination level [3].

In this work, we have developed a novel and specific endotoxin adsorbent using a synthetic amphipathic cationic peptide, S3Δ, which is derived from one of the LPS binding domains of horseshoe crab Factor C [6–8]. Factor C is highly sensitive to trace levels of LPS. LPS-induced activation of Factor C triggers a coagulation cascade in the *limulus* amoebocyte lysate [9–11]. At least two highly specific LPS binding sushi domains in Factor C have been characterized [12], and four peptides with high affinity for LPS [13] and antimicrobial activity [14] have been designed based on the sequences of the core LPS binding regions of these domains, of which S3Δ is one example. S3Δ also binds lipid A, the toxic portion of LPS, in a simple, stoichiometric and non-cooperative way with a Hill's coefficient of 0.91 [13]. This peptide is non-cytotoxic and non-haemolytic. A detailed profile of binding affinity of S3Δ for LPS under various pH and ionic strength conditions is presented in this study. As a potential candidate adsorbent in selective binding and removal of endotoxin from fluids and biological preparations, S3Δ was covalently conjugated to diaminodipropylamine (DADPA) immobilized agarose to develop a specific and selective affinity matrix. The capability of the resulting affinity matrix to remove LPS from liquid solutions was tested under various pH and ionic strength. The efficacy of this matrix in endotoxin removal was compared with other reported adsorbents.

2. Experimental

2.1. Materials

S3Δ (NH₂-HAEHKVKIKVKQKYGQFPQGTEV-TYTCSGNYFLM-COOH) was synthesized and purified by Genemed Synthesis, USA. An endotoxin-removing affinity matrix was developed by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a coupling agent to link S3Δ to DADPA-immobilized agarose gel (Pierce, USA). The

rationale for choosing DADPA-immobilized agarose matrix is based on the considerations of large pore size and hydrophilicity of agarose, to minimize non-specific binding and allow fast perfusion. Furthermore, the extended DADPA spacer arm of 12 atoms is useful for immobilizing small peptides which may otherwise be sterically hindered upon immobilization. This allows more space for the peptide–LPS interaction. EDC was used to couple the C-terminal carboxyl of the S3Δ peptide to the amine group of DADPA, forming very stable amide bonds. For comparison of efficiency of LPS removal, Detoxi-Gel (Pierce) was used.

The LAL Kinetic-QCL assay kit was from BioWhittaker, USA. LPS (from *Escherichia coli* 055:B5), lipid A in 1,4'-diphosphoryl form (from *E. coli* F-583) and sodium deoxycholate (DOC) were from Sigma. Fluorescein isothiocyanate (FITC)-labeled LPS (from *E. coli* 055:B5) was purchased from List Biological Labs., USA. Bovine serum albumin (BSA), ovalbumin and chymotrypsinogen A were purchased from Pharmacia Biotech. Insect cell culture medium Sf-900 II SFM was from Gibco BRL, USA. All other chemicals were of analytical-reagent grade from Sigma. Pyrogen-free water used for buffer preparations was from Baxter Healthcare, Australia.

2.2. Measurement of the binding affinity of S3Δ peptide with lipid A under different conditions

Surface plasmon resonance (SPR) sensorgrams were recorded to monitor the binding between S3Δ and lipid A under different conditions, in real time mode on a BIAcore 2000 biosensor instrument. Lipid A at 1.0 mg/ml in pyrogen-free water was immobilized on a HPA sensor chip (Pharmacia, Sweden) according to the manufacturer's specification. Stock solution of S3Δ at 1 mM in pyrogen-free water was diluted with different buffers to a series of concentrations, and injected into the flow cell at a rate of 30 μl/min, using the diluent as running buffer. The binding response was measured as a function of time, and kinetic constants were calculated from the sensorgrams using BIA evaluation software version 3.1 (Biacore, Sweden). For regeneration of the chip surface, the bound peptide was removed by injection

of 100 mM of NaOH solution for 1 min or in 1 min pulses until baseline SPR was achieved.

2.3. Covalent conjugation of S3Δ to DADPA-immobilized agarose beads

DADPA-immobilized agarose was used to prepare the S3Δ-affinity matrix. S3Δ was dissolved at 2 mg/ml in conjugation buffer [0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.9% NaCl, pH 4.7] and used for conjugation by EDC with DADPA-agarose in a column, following the procedure of the supplier. After 3 h at room temperature, the column was drained and the flowthrough and subsequent eluent fractions of 1 ml each were collected. The absorbance at 280 nm ($A_{280\text{ nm}}$) was measured to calculate the total amount of peptide immobilized to the matrix. After regeneration with 5 column volumes of 1% DOC and washing with pyrogen-free water, the column or beads were ready for use. When not in use, the peptide-conjugated beads were stored at 4°C with 0.02% of sodium azide.

2.4. Fluorescence microscope observation of LPS binding

The binding of FITC-labeled LPS to S3Δ conjugated affinity beads was observed under fluorescence microscopy. A System Microscope BX60 (Olympus, Japan) connected with a video camera JVC KY-F55BE (JVC, Japan) to a personal computer installed with image software AcQuis (Syncope, UK), was used for the FITC fluorescence observation and digitalized picture taking. Blue light was used for excitation with the filter cut-off at 380 nm. All digital pictures were recorded at same equipment settings.

2.5. LPS adsorption assay

After equilibration in the appropriate buffer, the LPS-binding capacity of S3Δ coupled beads was tested batchwise, under different conditions of ionic strength and pH. An aliquot of 500 μl of standard LPS or FITC-LPS solution, in the absence or presence of 0.5 mg/ml of a protein (either BSA, ovalbumin, or chymotrypsinogen A), was incubated with 50 μl of wet affinity beads, in rotation for 2–3 h at

room temperature. The mixtures were then centrifuged at 100 g for 1 min to pellet the beads. The supernatants were re-centrifuged at 10000 g for 10 min to fully pellet residual beads. LPS concentrations of the solutions before and after incubation with beads were measured to calculate the removal efficiencies. When proteins were involved in the adsorption assay, the protein concentrations before and after incubation were also measured to calculate the protein recovery.

2.6. Quantification of LPS and proteins

LPS was quantified either by the LAL assay, which involved a chromogenic kinetic test using a pyrogen-free 96-well microassay plate [12], or by fluorometry with a luminescence spectrometer LS-50B (Perkin-Elmer) when FITC-labeled LPS was used. In fluorometry, samples in microassay plate were excited at 490 nm with a slit of 2.5 nm, and the fluorescence intensity at 525 nm was measured at emission slit of 2.5 nm; 515 nm filter was used to reduce background emission. The protein concentration in sample solutions was determined by absorbance at 280 nm with a spectrophotometer DU 650 (Beckman).

3. Results

3.1. Affinity of S3Δ for lipid A measured by real time interaction on the BIAcore 2000

S3Δ is a basic (isoelectric point, *pI* 9.6) and amphipathic peptide [12]. The binding affinity of S3Δ-peptide with lipid A, was measured under various conditions of pH and ionic strength by SPR technology in real time mode on the BIAcore 2000. Fig. 1 shows a series of sensorgrams obtained by injecting the peptide at different concentrations into the flow cell which was coated with lipid A, while 0.4 M NaCl was used for both the solvent of the peptide and running buffer. Sensorgrams under various buffer conditions were obtained in an identical way (data not shown). Kinetic constants (Table 1) were calculated from the sensorgrams by fitting with a 1:1 Langmuir model [12].

In the low salt buffers, at identical ionic strength,

pH significantly affects the affinity, which ranges from the lowest K_D of $2.3 \cdot 10^{-9} M$ at pH 5.0 to the highest K_D of $5.6 \cdot 10^{-6} M$ at pH 9.1. Increase in pH decreases the binding rate constant k_a (from the highest $1.4 \cdot 10^3 M^{-1} s^{-1}$ at pH 5.0 to the lowest $22 M^{-1} s^{-1}$ at pH 9.1), with a concomitant increase in the dissociation rate (from the slowest $3.2 \cdot 10^{-6} s^{-1}$ at pH 5.0 to the fastest $1.2 \cdot 10^{-4} s^{-1}$ at pH 9.1). This shows that the initial driving force for the binding is via electrostatic interaction, which is strengthened by lowering of pH because of increase in the cationicity of S3Δ. However, while increasing the ionic strength (from 0 to 0.4 M NaCl), only an insignificant half order of magnitude difference in K_D was observed, between the lowest K_D ($4.3 \cdot 10^{-7} M$, at 50 mM NaCl) and the highest K_D ($2.1 \cdot 10^{-6} M$, at 400 mM). Also there is no correlation between the increase of ionic strength with the change of the rate constants (k_a , k_d). Since electrostatic and hydrophobic interactions are the major forces that influence the affinity of LPS and peptide, this indicates a significant contribution of hydrophobic interaction, which compensates for the weakening of electrostatic interaction due to salt, and further stabilizes the binding of S3Δ with lipid A. The importance of hydrophobic interactions between the acyl chains of LPS and endotoxin-binding peptide has been verified by Frecker et al. [15].

Hence, peptide S3Δ, which is designed according to the LPS binding domain of Factor C, has high affinity for lipid A, over wide pH and ionic strength. Under our tested conditions, the lowest dissociation constant, K_D of $2.3 \cdot 10^{-9} M$ was observed in 20 mM

NaOAc, pH 5.0. Even the highest K_D of $5.6 \cdot 10^{-6} M$ at pH 9.1 (Table 1), is still comparable to antibody–antigen affinity. This high affinity results from a synergism between electrostatic and hydrophobic interactions [4] and possibly, also the structural adaptation of peptides upon binding with LPS [13].

3.2. LPS affinity matrix preparation and regeneration

Based on the versatility of S3Δ in binding lipid A under various pH and ionic strength, its application in LPS removal from solution has led us to prepare S3Δ conjugated affinity matrix for LPS. Using 2 mg/ml of peptides, coupling efficiency of 50–70% was routinely achieved. The affinity beads strongly adsorb FITC-labeled LPS in water (Fig. 2a), resulting in almost 100% of LPS-free solution. The beads used can be efficiently regenerated by mild conditions of 1% DOC (Fig. 2b) or 2 M NaCl (Fig. 2c). There was virtually complete dissociation of FITC-LPS from affinity beads.

In a batchwise assay, FITC-LPS bound to beads at the concentration of 5 μg/ml can be removed at 75% efficiency from beads by using 10 volumes of 1% DOC in a single incubation, for 1–2 h at room temperature. Using 10 volumes of 2 M NaCl, 80% regeneration efficiency was achieved. The regenerated affinity beads were tested for its repeated usage in LPS binding. Under the same condition of 20 mM Tris, pH 6.8, 50 mM NaCl, the beads that were used once at the loading amount of 5 μg/ml of FITC-LPS, were compared with the freshly prepared beads.

Table 1
Affinity of S3Δ for lipid A at different pH and ionic strength^a

Solvent condition	$k_a (M^{-1} s^{-1})$	$k_d (s^{-1})$	$K_A (M^{-1})$	$K_D (M)$
Water	$4.1 \cdot 10^2$	$2.4 \cdot 10^{-4}$	$1.7 \cdot 10^6$	$5.9 \cdot 10^{-7}$
50 mM NaCl	$1.8 \cdot 10^3$	$7.8 \cdot 10^{-4}$	$2.3 \cdot 10^6$	$4.3 \cdot 10^{-7}$
200 mM NaCl	$3.7 \cdot 10^2$	$4.0 \cdot 10^{-4}$	$9.3 \cdot 10^5$	$1.1 \cdot 10^{-6}$
400 mM NaCl	$3.3 \cdot 10^2$	$6.8 \cdot 10^{-4}$	$4.9 \cdot 10^5$	$2.1 \cdot 10^{-6}$
20 mM NaAc, pH 5.0	$1.4 \cdot 10^3$	$3.2 \cdot 10^{-6}$	$4.4 \cdot 10^8$	$2.3 \cdot 10^{-9}$
20 mM Tris–HCl, pH 6.8	$6.5 \cdot 10^2$	$7.2 \cdot 10^{-6}$	$9.0 \cdot 10^7$	$1.1 \cdot 10^{-8}$
20 mM Tris–HCl, pH 9.1	22	$1.2 \cdot 10^{-4}$	$1.8 \cdot 10^5$	$5.6 \cdot 10^{-6}$

^a Binding of peptide S3Δ with diphosphoryl lipid A (*E. coli* F-583) immobilized on HPA chip surface was monitored in real time mode on BIAcore 2000. Affinity constants were calculated from the sensorgrams using a 1:1 (Langmuir) binding model to fit with BIA evaluation 3.1 (k_a and k_d are rate constants; K_A and K_D are equilibrium constants).

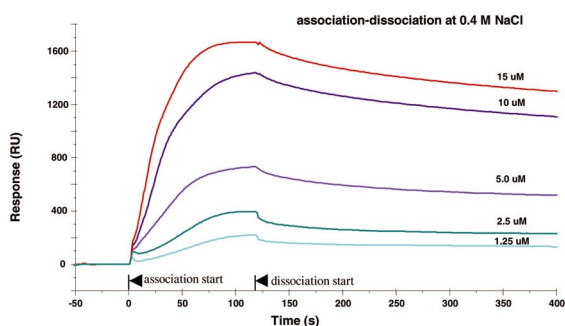


Fig. 1. SPR sensorgrams obtained on the BIAcore 2000 using a lipid A (*E. coli* F-583)-coated HPA chip show the real time biointeraction between S3Δ and lipid A in the presence of 0.4 M NaCl. S3Δ at a series of indicated concentrations was injected into the flow cell at a flow-rate of 30 μ l/min for 2 min.

In both cases, an LPS removal efficiency of \sim 80% was maintained.

3.3. Characteristics of LPS binding by S3Δ affinity beads

Since SPR analysis showed that S3Δ exhibited high affinity for lipid A under wide pH and ionic strength, the robustness of LPS binding of the affinity beads was tested under various ionic strength and pH.

Fig. 3a shows that, the LPS binding ability of S3Δ decreases with increase in ionic strength of the buffer, again indicating that the initial interaction of LPS with the peptide is mainly driven by electrostatic interaction between the negatively charged phosphate groups of lipid A moiety and positively charged lysine residues of S3Δ. From 50 to 400 mM NaCl, the LPS removal efficiency was reduced by a mere 25%. This indicates significant contribution of the hydrophobic interaction between LPS and peptides, as proven by BIAcore experiments using lipid A, thus suggesting the potential of S3Δ to make an affinity matrix which would be less dependent on low salt condition than other traditional anion exchanger like DEAE [3].

As shown in Fig. 3b, increasing the pH from 4 to 9.1 did not significantly alter the LPS binding efficiency. This indicates a wide pH tolerance of the peptide affinity beads. From pH 4 to 6.8, which is below pK_2 of 8.2 of the lipid A phosphate group

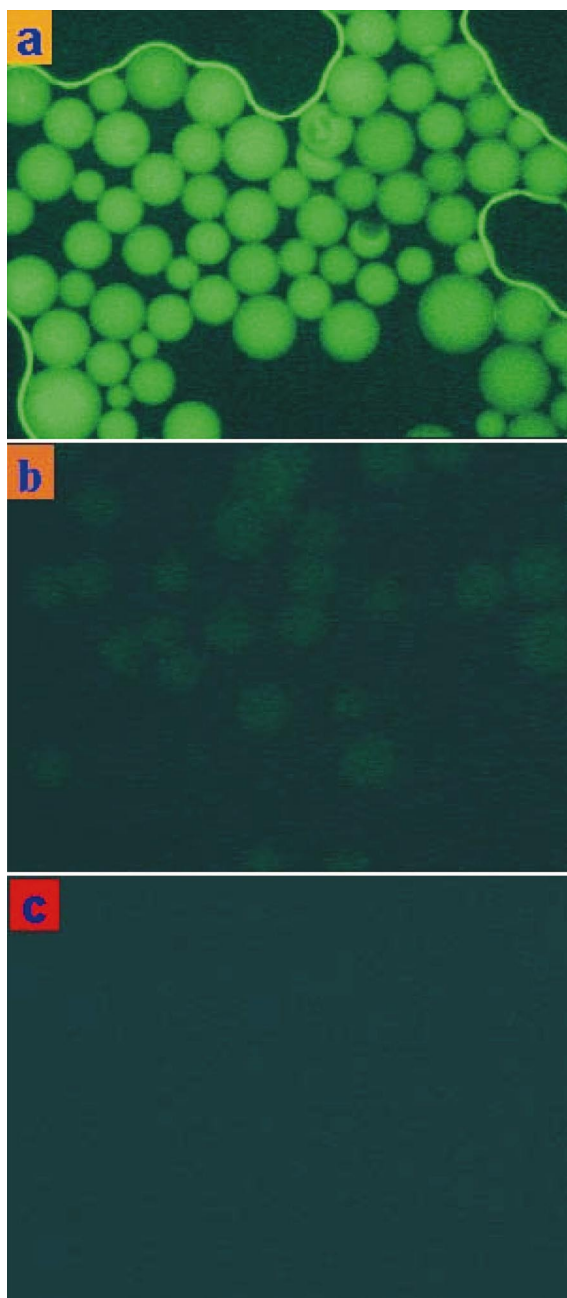


Fig. 2. Fluorescence microscopy shows the binding of LPS to S3Δ affinity beads followed by its dissociation from the beads by 1% of DOC or 2 M of NaCl. Blue light (cut-off at 380 nm) was used for excitation. (a) Beads with bound FITC-LPS; (b) beads after treatment with 1% DOC; and (c) beads after treatment with 2 M NaCl. All digital pictures were taken at the same equipment settings.

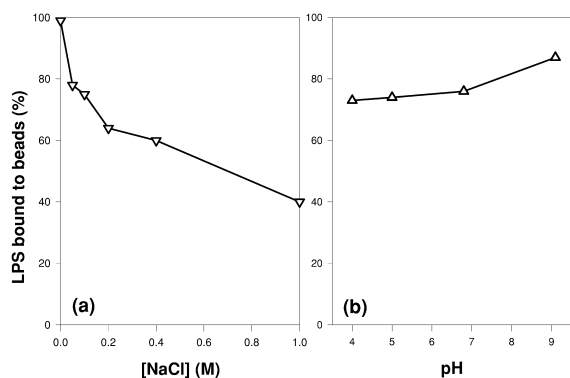


Fig. 3. LPS binding by peptide S3 Δ affinity beads under different (a) pH and (b) ionic strength. Aliquots of 0.5 ml of 5 μ g/ml of FITC-LPS in a buffer at indicated pH (either 20 mM sodium acetate, pH 4.0 and 5.0, containing 50 mM NaCl or 20 mM Tris-HCl, pH 6.8 and 9.1 containing 50 mM NaCl), or ionic strength (20 mM Tris-HCl, pH 6.8, containing indicated concentrations of NaCl), were incubated with 50 μ l of affinity beads in rotation for 2–3 h. Fluorescence intensities of the samples before and after treatment with beads were measured to calculate the LPS binding efficiencies.

[16], the net negative charge of LPS would not change while the net positive charge of peptide would be reduced from 6.28 to 3.24 (calculated using DNAMAN version 4.15 from Lynnon BioSoft). The increase in pH would expectedly lead to the weakening of electrostatic interaction between the negatively charged phosphate groups of LPS and the positively charged peptides, and hence decreases the LPS binding capacity of the peptide. However, this was not observed, and it strongly indicates that the hydrophobic interaction between LPS and peptide contributes significantly to the binding. From pH 6.8 to 9.1, the phosphate group in lipid A would increase one net negative charge, and the positive charge of the peptide S3 Δ would further reduce from 3.24 to 1.12. We observed a significant increase by 15% in LPS removal when pH was shifted from 6.8 to 9.1. This is attributable to the enhanced repulsive force between phosphate groups, thus reducing the aggregation of LPS. Based on our results, the S3 Δ affinity beads can be efficiently employed for LPS removal over a wide pH range.

3.4. Selective removal of LPS from protein solutions

While the electrostatic and hydrophobic interactions between proteins and LPS may complicate the removal of LPS from protein solutions, and different solvent conditions may render further complexity, the actual output would be difficult to predict. Different process conditions need to be tested for optimization of a target protein solution, often at the expense of compromising protein recovery and LPS removal efficiency [3]. In this work, 0.5 mg/ml of BSA (*pI* 4.7), ovalbumin (*pI* 4.6) and chymotrypsinogen A (*pI* 9.5) spiked with FITC-labeled LPS at 5 μ g/ml ($5 \cdot 10^4$ EU) were used to test the ability of S3 Δ conjugated agarose beads for selective removal of LPS from protein solutions.

As shown in Fig. 4, when pH was fixed at 6.8, the LPS removal efficiency from all three protein solutions consistently decreased with increase in salt concentration. While the recovery efficiency of a basic protein, chymotrypsinogen A, was maintained at 90% over the change of ionic strength, that of acidic BSA and ovalbumin drastically dropped with the decrease of salt concentration. For chymotrypsinogen A, low salt concentration of 50 mM NaCl enabled 80% LPS removal while achieving a concomitant protein recovery of 90%. Thus, the lower the ionic strength, the more efficient is the LPS removal from chymotrypsinogen solution. For BSA and ovalbumin, a compromise has to be made with neutral pH conditions and 200 mM NaCl to achieve optimum removal of LPS while maintaining 70% recovery of protein.

Using a fixed salt concentration of 50 mM, while changing the pH, the LPS removal efficiency from all protein solutions did not change significantly along the pH shifts (Fig. 5). This trend is similar to removal of LPS in Tris buffer (see Fig. 3), where 80% removal of LPS was achieved. While the recovery efficiency of basic protein, chymotrypsinogen A was maintained at 90% along the change of pH, that of acidic BSA and ovalbumin dropped drastically with the increasing pH. At pH 4 which is near the *pI* of BSA and ovalbumin, 80–90% of protein recovery can be reached without significant loss in LPS removal efficiency (~80%). Considering

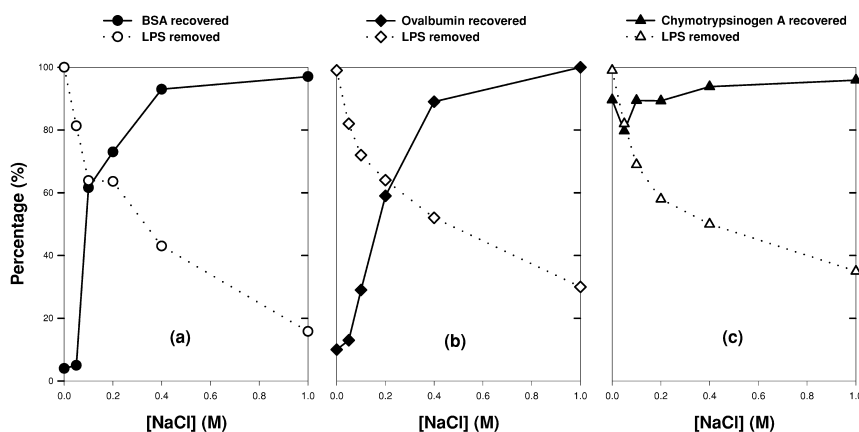


Fig. 4. LPS removal and protein recoveries from protein solutions under different ionic strength: (a) BSA, (b) ovalbumin and (c) chymotrypsinogen A. Aliquots of 0.5 ml of protein solutions at 0.5 mg/ml containing 5 μ g/ml of FITC-LPS in 20 mM Tris-HCl, pH 6.8 with indicated concentrations of NaCl were incubated with 50 μ l of affinity beads in rotation for 2–3 h. $A_{280\text{ nm}}$ and fluorescence intensities of the samples before and after treatment with beads were measured to calculate the LPS removal efficiencies and protein recoveries, respectively.

the intrinsic “sticky” property of BSA as a universal carrier molecule, this level of LPS removal is respectable. This efficiency can be further improved by the use of EDTA (see Section 3.5).

3.5. Removal of low problematic concentrations of LPS from various solutions

The pilot results presented above demonstrate that

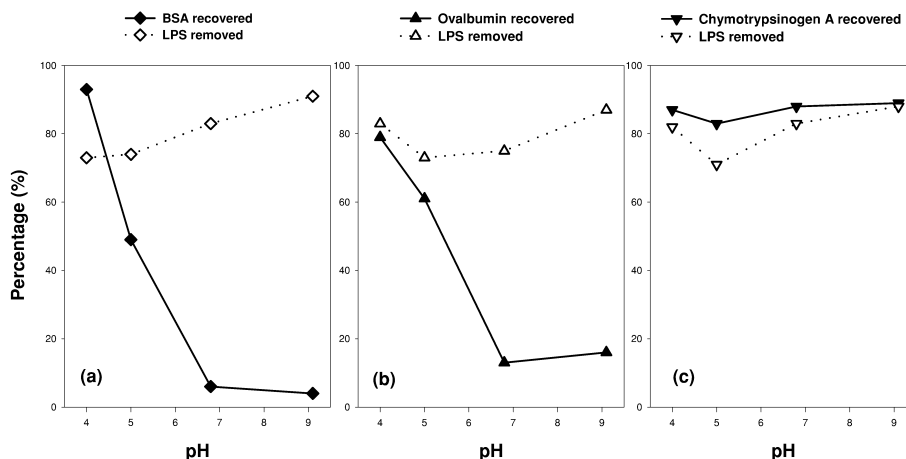


Fig. 5. LPS removal and protein recoveries from protein solutions under different pH conditions: (a) BSA, (b) ovalbumin and (c) chymotrypsinogen A. Aliquots of 0.5 ml of protein solutions at 0.5 mg/ml containing 5 μ g/ml of FITC-LPS in a buffer at indicated pH were incubated with 50 μ l of affinity beads in rotation for 2–3 h. $A_{280\text{ nm}}$ and fluorescence intensities of the samples before and after treatment with beads were measured to calculate the LPS removal efficiencies and protein recoveries, respectively. The buffers used were 20 mM sodium acetate at pH 4.0 and 5.0, and 20 mM Tris-HCl at pH 6.8 and pH 9.1. All buffers were supplemented with 50 mM NaCl.

S3Δ peptide affinity matrix can remove LPS from water and protein solutions, with good protein recovery, and it is less dependent on low ionic strength. Subsequently, we tested its performance at removing LPS at the usual problematic contamination level, which is reported to be up to 100 EU/ml for various biological preparations after the initial steps of purification [3]. Thus, we further tested buffers, protein solutions and a cell culture medium (Table 2).

For 20 mM Tris, pH 6.8 containing 50 mM NaCl and LPS ranging from 0.1 to 100 EU/ml, the LPS level was reduced to below the detection limit of 0.005 EU/ml from starting LPS level of 0.1, 1 and 10 EU/ml, and to 0.01 EU/ml from 100 EU/ml, in which case a clearance factor (CF) greater than 10^4 was achieved.

For 0.5 mg/ml of BSA and chymotrypsinogen A, spiked with 10 EU/ml of LPS, the LPS removal efficiencies were at CF of 2.7 and 59, respectively. However, in the presence of 5 mM EDTA the performance was improved by 10-fold for BSA and threefold for chymotrypsinogen A, where LPS levels were reduced to 0.3 and 0.06 EU/ml, respectively. The enhancement by EDTA is attributable to its “solubilization” effect on protein-bound LPS [3].

For the cell culture medium (Sf-900 II SFM), both spiked levels of 10 and 1000 EU/ml were reduced to below the detection limit 0.005 EU/ml, with no appreciable loss of medium components.

3.6. Comparison with commercially available gel matrix for endotoxin removal

A comparison of LPS removal efficiency between S3Δ peptide-affinity matrix and the commercially available endotoxin removing gel, with which polymyxin B is immobilized, was carried out by mini-column chromatography. Table 3 shows that while both gels exhibit a similar protein recovery of ~90%, the S3Δ peptide-affinity matrix displayed a superior LPS removal efficiency. Purification through S3Δ peptide-affinity column yielded a CF of 10^4 for the culture medium Sf-900 II SFM which contained a spiked level of 1000 EU/ml LPS. When spiked at 10 EU/ml, LPS was removed to below the detection limit. Using batchwise incubation with S3Δ peptide-affinity gel, purification of Sf-900 II SFM spiked with 1000 EU/ml LPS was further improved to below detection limit of 0.005 EU/ml. In comparison, Detoxi-Gel showed a much poorer performance.

Table 2
Removal of LPS from various solutions with problematic low levels of LPS contamination^a

	Tris buffer ^b		BSA ^b		Chym. A ^b		Medium ^b	
Before treatment (EU/ml)	100	10, 1, 0.1	10		10		1000	10
After treatment (EU/ml)	<0.01	<0.005 ^c	3.6	0.31 (+EDTA) ^d	0.17	0.06 (+EDTA) ^d	<0.005 ^c	<0.005 ^c
Clearance factor (CF)	>104	N/A	2.7	32	59	167	N/A	N/A

^a LPS adsorption experiments were carried out in a batchwise manner. Aliquots of 0.5 ml of each sample solution containing LPS at indicated concentrations were treated with 50 μl of wet affinity beads in rotation for 2–3 h. LPS concentration of samples before and after treatment was measured by LAL chromogenic assay. $A_{280\text{ nm}}$ was also measured to evaluate the recovery of components in the medium.

^b Tris buffer: 20 mM Tris–HCl, pH 6.8 containing 50 mM NaCl; BSA: BSA in 20 mM Tris–HCl, pH 4.0 containing 50 mM NaCl; Chym. A: chymotrypsinogen A, in 20 mM Tris–HCl, pH 9.1 containing 50 mM NaCl; medium: Sf-900 II SFM for insect cell culture, pH 6.5.

^c 0.005 EU/ml is the detection limit of LAL chromogenic assay.

^d EDTA at stock concentration of 0.5 M (pH 8.0) was added to the protein–LPS–bead mixture to achieve a final concentration of 5 mM before incubation.

Table 3

Comparison of LPS removal from cell culture medium Sf-900 II SFM by S3Δ peptide-affinity gel and Detoxi-Gel^a

Starting level of LPS (EU/ml)	After S3Δ peptide-affinity column (EU/ml)	After Detoxi-Gel column (EU/ml)
1000	0.10	661
10	<0.005	0.83

^a A column of 1 ml of S3Δ peptide-affinity gel with exactly the same dimensions as that of Detoxi-Gel endotoxin removing column was packed. After washing with 5 ml of 1% DOC and three times with 5 ml of water, the columns were loaded with 10 ml of cell culture medium Sf-900 II SFM containing 10 or 1000 EU/ml of LPS. After flowing twice through the respective columns by gravity, LPS concentration was measured by LAL chromogenic assay. $A_{280\text{ nm}}$ was also measured to evaluate the recovery of components in the medium.

4. Discussion

Owing to the pyrogenic effect of endotoxin, many pharmaceutical preparations have to meet a threshold of endotoxin contamination level, which is usually very low. For example, it is essential to eliminate LPS to at least a concentration lower than 100 pg/ml (~1 EU/ml) from fluids used for intravenous injection [5,17]. To date, there is no single general method available for the removal of endotoxin from solutions. Methods used for decontamination of water, such as ultrafiltration, have little effect on endotoxin levels in protein solutions [3]. Various techniques described in the patent literature are tailored to meet specific product requirements and not broadly applicable.

Generally, the high endotoxin concentrations in the starting materials used for the production of pharmaceuticals can be reduced to about 100 EU/ml or even lower by product purification steps without special treatment [3]. Even much lower remaining endotoxin contents may be realized. Bischoff et al. [18] purified recombinant α_1 -antitrypsin in a three-step procedure, employing ultrafiltration, anion-exchange and immobilized metal chelate affinity chromatography. As reviewed by Petsch and Anspach [3], most of those reported adsorbents can remove LPS with clearance factor from 10^3 to 10^5 at the $\mu\text{g/ml}$ of LPS feed level, but at the lower feed levels, the performance is usually not satisfactory, with either low clearance factor (5–200), or severe protein loss.

We have demonstrated that S3Δ affinity matrix can be used as an efficient and re-usable selective adsorbent for the removal of endotoxin from water, buffers, protein solutions and cell culture medium.

The ability of S3Δ peptide adsorbent to selectively adsorb LPS is attributable to its specific high affinity for LPS, with a K_D range from 10^{-6} to 10^{-9} M under tested range of pH and ionic strength. This selective matrix is particularly superior at the low problematic LPS contamination levels.

Although some published reports have documented adsorbents that can remove LPS with CF up to 10^5 at the loading level of $\mu\text{g/ml}$, at the low and problematic LPS contamination level (<100 EU/ml), the S3Δ affinity adsorbent displayed more superior performance. Furthermore, having no requirement for low ionic strength and yet, wide pH tolerance, make S3Δ-peptide affinity matrix an excellent novel LPS removal adsorbent with high selectivity and specificity. Unlike other reported adsorbents which are often toxic, S3Δ lacks cytotoxicity and it is non-haemolytic [12], thus further supporting its application in the pyrogen clean-up industry.

Acknowledgements

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