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Removal of lipopolysaccharides from protein–lipopolysaccharide complexes by nonflammable solvents

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Abstract

During the recovery of recombinant proteins from gram negative bacteria, many of the methods used to extract proteins from cells release lipopolysaccharides (LPS, endotoxin) along with the protein of interest. In many instances, LPS will co-purify with the target protein due to specific or non-specific protein–LPS interactions. We have investigated the ability of alkanediols to effect the separation of LPS from protein–LPS complexes while the complexes are immobilized on ion exchange chromatographic resins. Proteins were complexed with fluorescently labeled LPS and bound to ion exchange resin. Alkanediol washes of the resins were preformed and the proteins eluted. Column eluates were monitored for LPS and protein by fluorescence and UV spectroscopy, respectively. Alkanediols were effective agents for dissociating LPS from protein–LPS complexes. The efficiency of LPS removal increased with increasing alkanediol chain length. The 1,2-alkanediol isomers were more effective than terminal alkanediol isomers in the separation of LPS from protein–LPS complexes, while the separation of LPS from protein–LPS complexes was more efficient on cation exchangers than on anion exchangers. In addition, it was noted during these investigations that the 1,2-alkanediols increased the retention time of the proteins on the ion exchange resins. Alkanediols provide a safer alternative to the use of other organics such as alcohols or acetonitrile for the separation of LPS from protein due to their lower toxicity and decreased inflammability. In addition, they are less costly than many of the detergents that have been used for similar purposes.

Keywords: Lipopolysaccharides; LPS; Endotoxin; Ion exchange chromatography; Alkanediol; Nonflammable solvent

1. Introduction

Lipopolysaccharides (LPS) are a major component of the outer membrane of gram negative bacteria. The endotoxic component of LPS is the lipid A portion. It is composed of 1,6-linked D-glucosamine residues that are substituted with up to six acyl chains and a core polysaccharide structure to which additional polysaccharide repeating units may be attached. Endotoxin is a potent activator of the innate immune system at low doses while at higher doses endotoxin induces a number of other physical reactions including septic shock and death [1]. Contamination of therapeutic products with endotoxins is therefore a primary concern for the manufacturers of such products.

Many recombinant proteins are produced in the gram negative bacteria *Escherichia coli*. The removal of LPS from these recombinant proteins can be a complicated but essential process especially if the proteins are destined for therapeutic uses. Many different processes have been developed for the removal of LPS from proteins based on the unique molecular properties of the endotoxin molecules. These include LPS affinity resins, two-phase extractions, ultrafiltration, hydrophobic interaction chromatography, ion exchange chromatography, and membrane adsorbers (reviewed in [2]). These procedures have varying degrees of success in the separation of LPS from proteins, which in a large part is dependent on the properties of the protein of interest.

During the course of many of our *E. coli* development projects, we have had the opportunity to test several of the published procedures for the removal of LPS from LPS binding proteins. Some of the procedures that we have examined

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include denaturing hydrophobic interaction chromatography (HIC) [3] and the use of ethanol, isopropanol [4], or detergent [5] washes of proteins immobilized on ion exchange chromatographic resins. Alcohol and detergent washes during ion exchange chromatography were effective in reducing the protein associated LPS levels while poor separation of the LPS from the proteins was observed by the denaturing HIC procedure, while the detergents (Zwittergent 3–12 or 3–14) were usually more effective washing agents than the alcohols (unpublished results). Improved LPS clearance was achieved when the LPS–protein complexes were bound to a cation exchange resin as opposed to an anion exchange resin though the washing procedures used to remove LPS were effective on both matrices (unpublished results). Even though the alcohol and detergent washes were successful at reducing the levels of LPS in the LPS–protein complexes, scaling up and implementing any of these procedures in a manufacturing setting has many challenges. The concentrations of ethanol and isopropanol required to reduce the LPS levels of the LPS binding proteins were greater than 50% (v/v). Solutions of ethanol and isopropanol at these concentrations are considered flammable liquids and as such impose many safety and operational restrictions. The detergents, even though they were also very effective at reducing the LPS levels, are relatively expensive, would add significant cost to a manufacturing process, and may effect the bioactivity of the protein of interest. Alternative chemicals were desired that could safely and cost effectively be used in place of the alcohols or detergents as washing agents for the separation of LPS from proteins during chromatographic unit operations. Ideally, these chemicals would be relatively inexpensive, well defined chemically, present minimal safety issues, and have minimal impact on the bioactivity of the protein in question when implemented into a process.

1,5-Pentanediol, 1,6-hexanediol, 1,7-heptanediol, and hexylene glycol have been used as eluents for reversed phase chromatographic resins [6–8]. They provide increased safety over the use of the common reversed phase eluents like acetonitrile, ethanol, and methanol since the alkanediols are all nonflammable compounds. These compounds are soluble in water and they are not cost prohibitive. Solutions of these compounds do have increased viscosity, which may limit their use in certain applications. Because these compounds have successfully replaced organic solvents, such as ethanol, isopropanol, and acetonitrile, in reversed phase chromatographic applications, the ability of alkanediols to replace these same solvents during ion exchange chromatography for the separation of LPS from proteins was investigated. Alkanediols were able to effect the separation of LPS from LPS–protein complexes while the complexes were immobilized on ion exchange chromatographic resins. 1,2-Alkanediols were more effective than terminal alkanediols. The larger alkanediols were more effective than the smaller alkanediols. LPS removal was more efficient on cation exchangers as opposed to anion exchangers. It was also noted that the 1,2-alkanediols increased the retention time of the proteins during both anion and cation exchange chromatography.

2. Experimental

2.1. Materials

Bovine albumin fatty acid free and low endotoxin (BSA, 66,400 Da, p*I* 5.56), bovine holo-transferring (75,800 Da, p*I* 6.5), lactoferrin from bovine milk (75,200 Da, p*I* 8.52), lysozyme from chicken egg whites (14,300 Da, p*I* 9.65), lipopolysaccharides from *E. coli* serotype 055:B5, and BSTFA were purchased from Sigma Chemical Co. (St. Louis, MO). Acetic acid, Tris (base), sodium hydroxide (NaOH), hydrochloric acid, sodium chloride (NaCl), ethanol, isopropanol, sodium dodecyl sulfate (SDS), and sodium phosphate dibasic 7-hydrate were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). 1,6-Hexanediol was from BASF Co. (Mount Olive, NJ). 1,2-Hexanediol, 1,2-butanediol, and Zwittergent 3–14 (Zw 3–14) were purchased from Fluka (Milwaukee, WI). 1,4-Butanediol and ethylene glycol were purchased from Aldrich (Milwaukee, WI). Phosphate buffered saline (PBS), 10×, was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). *E. coli* BODIPY® FL conjugate lipopolysaccharide, serotype 055:B5, (BODIPY–LPS) and EnzChek Lysozyme Assay Kit were purchased from Molecular Probes, Inc. (Eugene, OR). Pyrosol, *Limulus Amebocyte* Lysate (LAL) Pyrotell-T, LAL reagent water (LRW), and control standard endotoxin from *E. coli* 0113:H10 (CSE), were obtained from Associates of Cape Cod, Inc.(Falmouth, MA). SP Sepharose Fast Flow (SPFF) resin, Q Sepharose Fast Flow (QFF) resin, and HR 10/10 columns were from Amersham Biosciences (Piscataway, NJ). Clear polystyrene 96-well microtiter plates were from Associates of Cape Cod, Inc. (Falmouth, MA) and black 96-well microtiter plates from NUNC (Rochester, NY). β-Hydroxytetradecanoic acid, β-hydroxytridecanoic acid, β-hydroxyundecanoic acid, β-hydroxytridecanoate, β-hydroxytetradecanoate, and β-hydroxyundecanoate were purchased from Matreya, Inc. (Pleasant Gap, PA). Heptane was purchased from Spectrum (New Brunswick, NJ).

2.2. Methods

2.2.1. Viscosity measurements

Viscosities of the solutions were determined at 20 °C using a Brookfield Model 1 LVT Viscometer equipped with a ULA-Y adapter (Middleboro, MA) according to the manufacturers' directions.

2.2.2. LPS–protein complex formation

Protein stock solutions were prepared in the column equilibration buffers to a final concentration of 10–11 mg/ml. LPS O55:B5 stock solution was prepared in Milli-Q water and BODIPY–LPS stock solution was prepared in PBS

or column equilibration buffers to a final concentration of 1 mg/ml. This is approximately 100 μM final concentration based on a molecular weight of 10,000 Da for O55:B5 LPS.

The LPS–protein complexes were formed by adding 1 part LPS solution to nine parts protein solution, v/v, in a polypropylene tube. The tube was vortexed, wrapped in aluminum foil, and then incubated at room temperature for 16–72 h for BSA and transferrin or incubated at 37 °C for at least 4 h for lactoferrin.

2.2.3. LAL and BODIPY analysis

A SpectraMax Gemini XS microplate spectrofluorometer from Molecular Devices Co. (Sunnyvale, CA) was used for the BODIPY–LPS fluorescent microplate assay and a SpectraMax 190 microplate spectrophotometer (Molecular Devices) for the LAL kinetic turbidimetric assay (KTA). Reference materials were analyzed in triplicate and samples in either duplicate or triplicate. The results were plotted and analyzed using SOFTmax PRO software Version 3.1.1 (Molecular Devices).

2.2.3.1. BODIPY–LPS assay. Three-fold serial dilutions of the BODIPY–LPS stock solution were prepared from 0.03 to 0.81 μM (54–1458 ng). The analytical procedure of the BODIPY–LPS assay was a modification of the assay used by Yu and Wright [9] as follows. To each well of a black microtiter plate 20 μl of 15% SDS, prepared in Milli-Q water, followed by 180 μl of sample or standard were added. The plate was shaken for 10 s at 37 °C and read immediately in fluorescence mode. The optimal excitation (490 nm), emission (525 nm), and cut-off wavelengths (515 nm) were experimentally determined for the BODIPY–LPS. The assay for BODIPY–LPS demonstrated a linear range from 0.03 to 0.81 μM with a limit of detection of less than 0.01 μM (18 ng) and a limit of quantification of 0.03 μM (54 ng).

2.2.3.2. LAL kinetic turbidimetric assay. Prior to analysis samples were adjusted to a pH between 6 and 8 with Pyrosol, if needed. The CSE and Pyrotell-T were reconstituted with LRW. To each well of a clear polystyrene microtiter plate 100 μl of sample or standard and 100 μl of Pyrotell-T were added. For spiked samples, 5 μl of 2.00 EU/ml CSE was added to obtain 0.10 EU/ml CSE level. The plate was shaken for 10 s and data collected, every minute, in the kinetic mode at 405 nm for 1 h at 37 °C. The linear curve of CSE was from 0.03 to 1.00 EU/ml.

2.2.3.3. LPS analysis by gas chromatography (GC). Quantitative analysis of LPS by gas chromatography–mass spectrometry (MS) was based on the method by Mıelnıczuk et al. [10]. The analysis was performed on a 6890 gas chromatograph with a 5973 mass selective detector from Agilent (Foster City, CA). The column used was a DB-5MS column (30 cm × 0.25 mm i.d. ×0.25 μm film thickness) from J&W Scientific (Foster City, CA). The linear curve for surrogate and target compounds ranged from 1.6 to 100 pg/μl and the internal standard was kept constant at 50 pg/μl. Briefly, the method of sample preparation and GC–MS analysis were as follows. Samples and a known amount of surrogate, β-hydroxytridecanoic acid, were added to 5 ml glass reaction vials. Aqueous samples were hydrolyzed in 6N HCl at 90–100 °C overnight to liberate β-hydroxytetradecanoic acid from LPS. The fatty acids were extracted twice from the hydrolysate with heptane and the solvent layers pooled and dried under nitrogen. The fatty acids were methylated by incubation at 80–90 °C in 3N methanolic HCl for 30 min. Water was added to quench the reaction and then the methyl esters extracted twice with heptane. The solvent were pooled and dried under nitrogen. The methyl esters were derivatized by adding BSTFA/pyridine (2:1, v/v) and incubating at 80–90 °C for 15–20 min before undergoing the final drying step under nitrogen. Samples were reconstituted with heptane containing the internal standard, methyl-3-trimethylsilyl-undecananoic acid, at 50 pg/μl. Standards and samples were injected in splitless mode and at 1 μl injection volume. Initial oven temperature was held at 90 °C for 4 min and then ramped at 20 °C per minute to 250 °C followed by a 10 °C per minute ramp to 300 °C. The mass spectrometer was set for an EM offset voltage of 500 and the solvent delay at 5.2 min. Selective ion monitoring was used to monitor methyl-3-TMS-undecanoate at ions 175 and 273, methyl-3-TMS-tridecanoate at 11.0 min and ions 175 and 301, and methyl-3-TMS-tetradecanoate at 11.7 min and ions 175 and 315. Chromatograms were reported using Chemstation for MSD Productivity software.

2.2.4. Lysozyme assay

Lysozyme activity was determined using the EnzChek Lysozyme Assay Kit according to the manufacturers' instructions.

2.2.5. Chromatography

All chromatography was performed on ÄKTA explorer 100 FPLC systems (Amersham Biosciences) at ambient temperature. Column flow rates were between 200 and 300 cm/h except during the alkanediol washes when the flow rates were dropped to 150–200 cm/h to minimize the increase in system back pressure due to the increased viscosity of the alkanediol solutions. Chromatograms were reported using Unicorn software Version 3.21 or 4.0. The resins were packed in 1 cm diameter columns to bed heights of 7–11 cm. The ÄKTA systems and columns were sanitized either with 0.5N NaOH for 60–120 min or with 0.1N NaOH for greater than 16 h before each chromatographic run. The columns and ÄKTA systems were rinsed with Milli-Q water just prior to system equilibration with the appropriate buffers.

Alkanediols, ethanol, and isopropanol were prepared as v/v solutions with the same chemical composition and pH as the equilibration buffer for a given analysis. 1,6-Hexanediol and Zwittergent 3–14 were prepared as w/v solutions.

2.2.5.1. Cation exchange chromatography. For transferrin studies, a SP Sepharose Fast Flow column was charged with

100 mM acetate, 1 M NaCl, pH 5, and equilibrated with 100 mM acetate, pH 5. After loading, the column was washed with the equilibration buffer and then eluted with 50 mM sodium phosphate, 1 M NaCl, pH 7.5. When an organic or detergent wash was performed, it was applied after the initial wash step and was for 6 column volumes (CV) unless otherwise stated. This wash was followed by a second wash with equilibration buffer to remove the organic or detergent prior to elution.

BSA studies were identical to those for transferrin except that the pH of all chromatography buffers was 4.5. When 1,2-hexanediol was used as the washing agent, the eluent was changed to 50 mM sodium phosphate, 1 M NaCl, pH 7.5.

For the lactoferrin studies, the columns were charged with 1 M sodium chloride, 20 mM sodium phosphate, pH 7.5, equilibrated in 20 mM sodium phosphate, pH 7.5, and eluted with 1 M sodium chloride, 20 mM sodium phosphate, pH 7.5.

For the lysozyme studies the columns were charged with 1 M NaCl, 20 mM Tris, pH 8.0, equilibrated in 50 mM Tris, pH 8.0, and eluted with 1 M NaCl, 20 mM Tris, pH 8.0.

2.2.5.2. Anion exchange chromatography. For the BSA studies, a Q Sepharose Fast Flow column was charged with 50 mM Tris, 1 M NaCl, pH 8.0 and equilibrated with 50 mM Tris, pH 8.0. After loading, the column was washed with equilibration buffer. BSA was eluted with 25 mM Acetate, pH 4.5, and LPS with 25 mM Acetate, 1 M NaCl, pH 4.5. When an alkanediol wash was performed, it was inserted after the initial wash step and was for 6 CV. This wash was followed by a second wash with equilibration buffer to remove the alkanediol.

3. Results and discussion

Lactoferrin, transferrin, and BSA have all been shown to bind LPS [11–13]. Lysozyme was used to assess the effects of the washing agents on enzyme activity.

Table 1 summarizes some of the physical properties of the alkanediols used in this study. Also included, for comparison, are ethanol and isopropanol, which have been used for LPS removal in other processes [4].

3.1. Separation of LPS–protein complexes by organics and detergents

3.1.1. SP Sepharose Fast Flow chromatography of LPS and LPS–protein complexes

The LPS elution profiles of LPS by itself and LPS–BSA complexes on SP Sepharose Fast Flow resin were determined by LAL-KTA analysis of selected column fractions. When LPS was chromatographed by itself, the LPS was detected primarily in the wash-unbound fraction, 53% of the loaded amount, as expected. Only 0.3% of the LPS loaded was associated with the eluate fraction. Chromatography of the LPS–BSA complexes resulted in the majority of the LPS being detected in the BSA eluate fraction, 58% of the loaded LPS, while only 8% of the LPS loaded was recovered in the wash-unbound fraction. This result confirms the LPS binding property of BSA [13] and demonstrates that the BSA–LPS complexes that were prepared are stable under cation exchange chromatography conditions employed.

The LAL KTA is a laborious and costly assay to determine the distribution of LPS in the column fractions. A fluorescent based assay for LPS was developed to monitor the column fractions. This assay used fluorescently tagged LPS, BODIPY–LPS, in place of the non-labeled LPS, which allowed for the quick analysis of the column eluates by fluorescence spectroscopy. The fluorescence of the BODIPY marker in the BODIPY–LPS conjugate has been shown to be quenched when the LPS is complexed with itself or protein. Addition of SDS to the sample disrupts the LPS–LPS or LPS–protein complexes and results in an increase in fluorescence [9]. The assay was developed as a microtiter plate based assay that allowed for the quick and quantitative analysis of BODIPY–LPS in the chromatography fractions.

To determine if the BODIPY marker interfered with BSA–LPS complex formation or behaved differently during cation exchange chromatography the preceding analysis was repeated using BODIPY–LPS and BODIPY–LPS–BSA

Table 1
Physical properties of alkanediols used in this study

Compound	Boiling point (°C)[a]	Melting point (°C)[a]	Flash point (°C)[a]	Explosion limits[b], air		Autoignition (°C)[b]
				Lower (%)	Upper (%)	
1,2-Hexanediol	223	NA	122	NA	NA	390
1,6-Hexanediol	250	45	147	6.6	16	319
1,5-Pentanediol	242	−16	129	1.4	13.2	NA
1,2-Butanediol	194	−50	93	2.4	13.5	390
1,4-Butanediol	230	16	121	1.95	18.3	420
1,3-Propanediol	214	−27	131	NA	NA	400
1,2-Ethanediol	195	−13	111	3.2	15.3	400
Ethanol	78.3	−114.1	12	3.3	19	363
Isopropanol	82.4	−88.5	12	2.5	12	460

NA, not available.

[a] Data obtained from CabridgeSoft Corp. at Chemfinder.com.

[b] Data from Material Data Safety Sheets.

complexes. The elution profiles of BODIPY–LPS and BODIPY–LPS–BSA complexes were similar to the elution profiles of LPS and LPS–BSA complexes above. For BODIPY–LPS, 82% of the BODIPY–LPS loaded was in the wash-unbound fraction and 5% in the eluate fraction and for the BODIPY–LPS–BSA complexes 3% of the loaded BODIPY–LPS was in the wash-unbound and 90% was in the eluate. This demonstrates that the BODIPY marker does not interfere with the ability of BSA to bind LPS and that the BODIPY group does not alter the chromatographic profile of the LPS. Recovery of the BODIPY ranged between 70 and 90% under these chromatographic conditions. The BODIPY–LPS assay has a variability of approximately ±5%. The variability observed in the recovery of BODIPY–LPS from the chromatography process was dependent on how the fractions were taken and the accuracy of the volume measurements of the fractions.

Similar results were obtained when transferrin was substituted for BSA. Fig. 1 shows typical SP Sepharose Fast Flow elution profiles for BODIPY–LPS (A) and the BODIPY–LPS–transferrin complex (B) both by fluorescence and optical density at 280 nm. BODIPY–LPS alone (Fig. 1A) elutes primarily in the wash-unbound fraction as determined by fluorescence and virtually no signal at 280 nm is detected. The BODIPY–LPS–tranferrin complex demonstrates co-elution of the protein, by A280, and the BODIPY–LPS, by fluorescence, demonstrating the complex

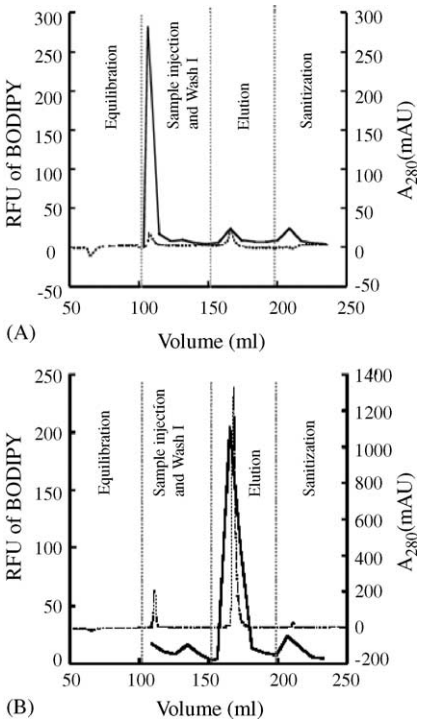

Fig. 1. SP Sepharose Fast Flow elution profiles of BODIPY–LPS and BODIPY–LPS–transferrin complexes. Chromatograms for (A) BODIPY–LPS and (B) BODIPY–LPS–transferrin. The dotted line is UV signal at 280 nm and the solid line corresponds to BODIPY fluorescence expressed in relative fluorescence units (RFU).

formation and its stability under the chromatographic conditions.

3.1.2. Reduction of LPS from LPS–protein complexes by alkanediols during SP Sepharose Fast Flow chromatography

Initial experiments examined the potential of a 50% 1,6-hexanediol wash step to reduce the amount of BODIPY–LPS complexed with BSA during cation exchange chromatography on SP Sepharose Fast Flow. A 3 CV wash with 1,6-hexanediol preceding BSA elution lowered the amount of BODIPY–LPS complexed with BSA by about 21%. Increasing the length of the 1,6-hexanediol wash step from 3 CV to 6 CV improved the removal of BODIPY–LPS from the BSA complex to about 49%. An additional 3 CV increase in the 1,6-hexanediol wash step to 9 CV only provided marginal improvement (51%) in BODIPY–LPS removal. All additional experiments were carried out with a 6 CV alkanediol wash step.

The effectiveness of a series of alkanediols to remove LPS from proteins while the proteins were bound to ionic solid supports were compared to those of ethanol, isopropanol, and Zwittergent 3–14, which have been shown to be effective in reducing the LPS content of protein bound LPS [4,5]. A SP Sepharose Fast Flow column was loaded with the BODIPY–LPS–transferrin complex. The column was washed with six column volumes of a 50% alkanediol solution and then eluted. Fractions were collected and assayed for BODIPY–LPS (Fig. 2). As the chain length of the alkanediol was increased from four to six carbons, the fluorescence of the alkanediol wash fractions increased while the fluorescence of the eluate fractions decreased. This demonstrated that alkanediols removed BODIPY–LPS from the transferrin complex and that the efficiency of the removal was dependent on the alkanediol structure. Figs. 3 and 4 illustrate the effects of alkanediol structure on BODIPY–LPS removal from transferrin and BSA, re-

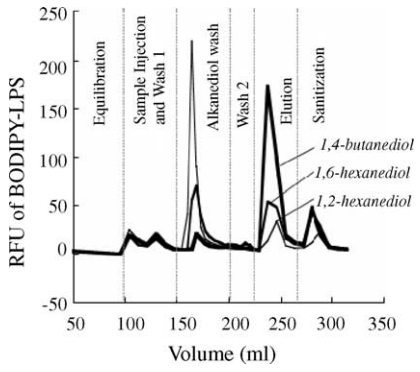

Fig. 2. BODIPY–LPS elution profiles of transferrin complexes on SP Sepharose Fast Flow in conjunction with alkanediol washes. BODIPY–LPS–transferrin complexes were generated, loaded onto a SP Sepharose Fast Flow column and the column washed with 50% solutions of 1,4-butanediol, 1,6-hexanediol, or 1,2-hexanediol. Following a wash to remove the alkanediol, transferrin was eluted as described in Section 2.2. Sanitization between runs was with 0.5N NaOH.

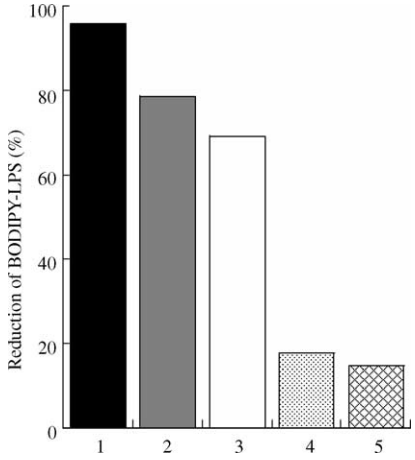

Fig. 3. The Reduction of BODIPY–LPS from transferrin complexes in SP Sepharose Fast Flow eluates by alkanediols. Chromatography was as described in Fig. 2. Zero percent reduction corresponds to a control run without an alkanediol wash (1) 1,2-hexanediol; (2) 1% Zwittergent 3–14; (3) 1,6-hexanediol; (4) ethylene glycol; and (5) 1,4-butanediol.

spectively. BODIPY–LPS removal efficiency increased with increasing alkanediol chain length and the 1,2-alkanediol isomers were more effective than the terminal alkanediols at removing the BODIPY–LPS. 1,2-hexanediol was the most efficient compound tested and out performed the detergent and alcohols. 1,2-Butanediol and 1,6-hexanediol as well as 50% isopropanol and 75% ethanol reduced the BODIPY–LPS associated with transferrin to similar levels. Ethylene glycol and 1,4-butanediol were only marginally effective at reducing LPS from the BSA or transferrin complexes. The removal of BODIPY–LPS by the alkanediols was similar for both the transferrin and BSA complexes.

It was noted during the BSA experiments utilizing 1,2-hexanediol in the wash that BSA was not effectively eluted from the resin at a pH of 5 with 1 M NaCl. Increased retention

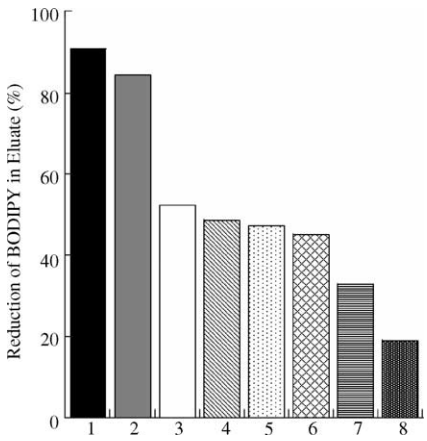

Fig. 4. The reduction of BODIPY–LPS from BSA complexes in SP Sepharose Fast Flow eluates by alkanediols. Chromatography was as described in Fig. 2. Zero percent reduction corresponds to a control run without an alkanediol wash (1) 1,2-hexanediol; (2) 1% Zwittergent 3–14; (3) 1,2-butanediol; (4) 1,6-hexanediol; (5) 50% isopropanol; (6) 75% ethanol; (7) 1,4-butanediol; and (8) ethylene glycol.

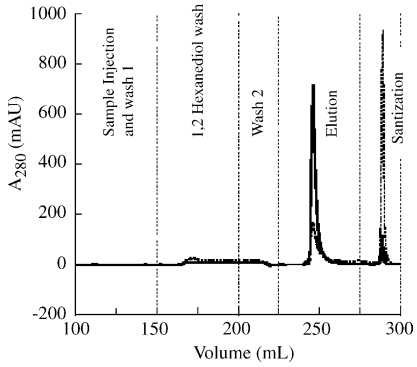

Fig. 5. Effect of 1,2-hexanediol concentration on the elution of BSA from SP Sepharose Fast Flow. BODIPY–LPS–BSA complexes were generated, loaded onto a SP Sepharose Fast Flow column and the column washed with 10% solution (dotted line) or 5% solution (solid line) of 1,2-hexanediol. Following a wash to remove the 1,2-hexanediol, elution was carried out with 1 M NaCl. Sanitization of the resin was with 0.5N NaOH.

of BSA was observed down to a 1,2-hexanediol concentration of 10%. At a 1,2-hexanediol concentration of 5%, BSA retention was not altered (Fig. 5). The alteration in retention times has been observed for other proteins during ion exchange chromatography in the presence of polyethylene glycol and other neutral polymers [14,15]. The changes in protein retention times were dependent on polyethylene glycol size, concentration, and the protein itself, whereas, ethylene glycol up to a concentration of 40% did not effect protein retention times [16]. It is worthwhile to note that in these instances the compounds under investigation were included in all the chromatography buffers. In the studies reported here, 1,2-hexanediol was only included in the initial wash buffer and was then removed by an additional wash step, without the 1,2-hexanediol, prior to elution of the protein.

Since 1,2-hexanediol was the most effective compound tested for removing BODIPY–LPS from both transferrin and BSA, the concentration dependence of the 1,2-hexanediol wash needed to effect this removal was investigated. The reduction of BODIPY–LPS in the SP Sepharose Fast Flow BSA eluate fraction was determined after 1,2-hexanediol washes containing 5%, 20%, and 50% 1,2-hexanediol. The 5% 1,2-hexanediol wash resulted in about a 55% decrease in the BODIPY–LPS eluting with the BSA while the reduction of BODIPY–LPS in the eluate by the 20% and 50% 1,2-hexanediol washes were comparable at approximately 96%.

In addition to transferrin and BSA, the removal of BODIPY–LPS from lactoferrin complexes by 1,6-hexanediol was also examined by the fluorescent BODIPY assay. The results of the analysis were confirmed by analysis of the samples for the LPS marker compound 3-OH-14:0 fatty acids by GC–MS. Table 2 summarizes the data. The GC–MS data support the fluorescent BODIPY data and demonstrates the ability of an alkanediol wash step, in this case 1,6-hexanediol, during the chromatography to reduce the levels of BODIPY–LPS in the lactoferrin eluate. Approximately an 87% reduction of BODIPY–LPS was observed by the

Table 2
SPFF chromatography elution profiles of BODIPY–LPS and BODIPY–LPS–lactoferrin complexes

Sample	BODIPY–LPS (% recovery) GC–MS assay/fluorescence BODIPY assay			
	Load	Wash-unbound	Diol wash	Eluate
BODIPY–LPS (no diol wash)	100 (GC–MS)	94	N/A[a]	5
	100 (fluor)	89	N/A	7
BODIPY–LPS–lactoferrin (no diol wash)	100	62	N/A	48
	100	60	N/A	46
BODIPY–LPS–lactoferrin (+diol wash)	100	63	ND[b]	4
	100	66	26	6

BODIPY–LPS–lactoferrin complexes were generated, loaded onto a SP Sepharose Fast Flow column. For runs that included a 50% solution of 1,6-hexanediol, a wash to remove the 1,6-hexanediol was included prior to elution of lactoferrin as described in Section 2.2. Column fractions were assayed for 3-OH-14:0 fatty acids by GCMS and BODIPY as described in Section 2.2.

[a] N/A, not applicable.

[b] ND, could not be determined by GC–MS.

BODIPY fluorescence assay and a 91% reduction in the LPS marker by the GC–MS assay.

During the chromatographic runs, a rise in the system back pressure was noted when the alkanediol washes were applied. The viscosities of each organic solutions and the Zwittergent solution, prepared in 100 mM Acetate, pH 4.5, were measured (Fig. 6). The viscosity of the alkanediols increased with carbon chain length and the viscosity of the 1,2-alkanediol isomers were slightly less than the terminal alkanediol isomers. The increased viscosity of the alkanediol solutions may present some difficulties in scale-up. Column flow rates may have to be adjusted to maintain suitable system pressure for the equipment in use. Being able to use lower concentrations of the alkanediols to remove LPS from the protein–LPS complexes would partially alleviate this problem. For example, 20% and 50% 1,2-hexanediol washes effectively reduce the BODIPY–LPS to approximately the same levels for BSA complexes as indicated above. The viscosity of 20% 1,2-hexanediol is about one third that of 50% hexanediol, 2.6 Cp compared to 7.5 Cp.

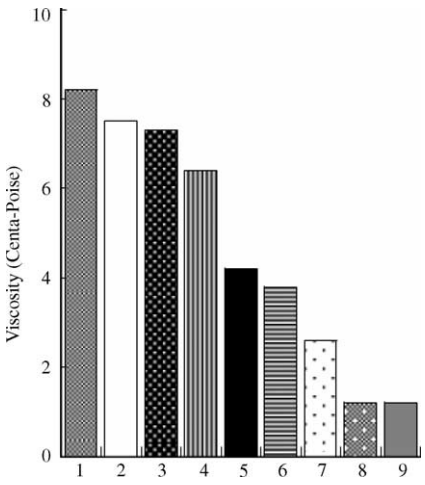

Fig. 6. Viscosities of alkanediol, isopropanol, ethanol, and Zwittergent solutions in 100 mM Acetate, pH 4.5. All solutions were prepared with 100 mM acteate buffer, pH 4.5. (1) 50% 1,6-hexanediol; (2) 50% 1,2-hexanediol; (3) 50% 1,4-butanediol; (4) 50% 1,2-butanediol; (5) 50% ethylene glycol; (6) 50% isopropanol; (7) 75% ethanol; (8) 1% Zwittergent 3–14; and (9) 100 mM acetate, pH 4.5.

3.1.3. Reduction of LPS from LPS–protein complexes by alkanediols during Q Sepharose Fast Flow chromatography

Removal of LPS from LPS–protein complexes is more complex on anion exchange resins, especially for basic proteins. During cation exchange chromatography, the LPS, being negatively charged, is not attracted to the functional group of the resin and is washed out of the column during the alkanediol wash while the protein remains bound under the wash conditions. During anion exchange chromatography, the LPS and the protein both are retained by the resin's functional groups. Therefore, the complexes need to be disrupted and differential elution of the protein and LPS must occur.

The ability of 1,2-hexanediol to reduce the BODIPY–LPS levels of BSA–LPS complexes during anion exchange chromatography on Q Sepharose Fast Flow resin was investigated.

It was found that BSA was not effectively eluted from the anion exchange resin with 1 M NaCl after the application of a 1,2-hexanediol wash, as was observed for BSA on the cation exchange resin. Increasing the salt concentration of the elution buffer to effect the elution of BSA after the 1,2-hexanediol wash resulted in the co-elution of BODIPY–LPS with the BSA (data not shown). An alternative elution scheme was chosen based on pH. After the 1,2-hexanediol wash was complete and a wash out of the 1,2-hexanediol had occurred, the BSA was eluted at pH 4.5. After the elution of BSA, the resin was striped with a buffer at pH 4.5 containing 1 M NaCl. The strip conditions brought off the remainder of the BODIPY–LPS. Table 3 summarizes the effect of 1,2-hexanediol on the removal of BODIPY–LPS from BODIPY–LPS–BSA complexes. Inclusion of the 1,2-hexanediol wash reduced the BODIPY–LPS of the BSA eluate by about 70% with an apparent elution of the displaced BODIPY–LPS to the 1,2-hexanediol wash fractions. Recovery of BODIPY–LPS from the anion exchange resin, 43–75%, was not as high as the recovery obtained from the cation exchange resin, 70–90%. The lower recoveries were

Table 3
The QFF elution profiles of BODIPY–LPS and BODIPY–LPS–BSA complexes

Sample	BODIPY–LPS (% recovery)				
	Load	Wash-unbound	Diol wash	Elute	Strip
BODIPY–LPS (no diol wash)	100	1	NA[a]	0	42
BODIPY–LPS–BSA (no diol wash)	100	7	NA	17	52
BODIPY–LPS–BSA (+diol wash)	100	2	12	5	45

BODIPY–LPS and BODIPY–LPS–BSA complex were chromatographed on Q Sepharose Fast Flow with and without a column wash with 1,2-hexanediol. Column fractions were analyzed for BODIPY–LPS by the BODIPY assay as described in Section 2.2.

[a] NA, not applicable.

in part due to how the fractions were collected and their volumes determined but the main reason can most likely be attributed to the high affinity of the BODIPY–LPS for the anion exchanger and only a partial elution by the 1 M NaCl strip.

3.2. Alkanediol effect on lysozyme activity

Lysozyme was used to determine the effect of the washing agents on enzymatic activity and thereby, indirectly the denaturing effects of the washing agents during SP Sepharose Fast Flow chromatography. Lysozyme was chromatographed with and without a 6 CV 50% 1,6-hexanediol wash or 1,2-hexanediol wash and the column loads and eluates assayed for lysozyme activity using a fluorescence microplate lysozyme activity assay. The recovery of lysozyme activity in SP Sepharose eluates were 86.3% without a wash, 87.4% with the 1,6-hexanediol wash, and 90.5% with the 1,2-hexanediol wash demonstrating that the hexanediol washes had no detrimental effects on lysozyme activity. Seventy five percent ethanol, 50% isopropanol, and 1% Zwittergent 3–14 washes also had no effect on lysozyme activity. Recoveries of lysozyme activities were 95.7%, 93.7%, and 107.0%, respectively.

4. Conclusion

Alkanediols were shown to be effective agents for the separation of LPS from LPS–protein complexes during chromatography on ionic supports. Their effectiveness in reducing the protein complexed with LPS is dependent on (1) the size of the alkanediol, (2) the isomeric form of the alkanediol, (3) the length of the alkanediol wash, (4) the concentration of alkanediol, and (5) the type of ionic support used, cationic or anionic. Longer chain alkanediols are more effective than the shorter chains. The 1,2-isomers are more effective than terminal isomers. LPS removal increases with increasing alkanediol concentration. In addition, LPS removal is more efficient on cationic exchangers as compared to anionic exchangers. Alkanediols are non-flammable and as such are safer alternatives to alcohols, such as ethanol or isopropanol, which have also been used to remove LPS from protein–LPS complexes. One potential drawback to the use of alkanediols is the increased viscosities of their solutions. This can be minimized, to some degree, by the appropriate choice of alkanediol since the viscosity is dependent on alkanediol chain length, isomer type, and chain length.

In addition, it was observed that inclusion of a 1,2-hexanediol wash during the chromatography significantly increased the retention time of transferrin on SP Sepharose Fast Flow and BSA on Q Sepharose Fast Flow. This phenomenon has been observed for other proteins with other types of neutral polymers although in these cases the polymer was included in all chromatographic buffers [14,15].

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