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Efficient method for preparation of highly purified lipopolysaccharides by hydrophobic interaction chromatography

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Abstract

A method for the efficient preparation of highly purified lipopolysaccharides (LPSs) by hydrophobic interaction chromatography (HIC) has been developed. The procedure can be used for the purification of cell wall bound LPSs after hot phenol–water extraction and for the isolation of extracellular LPSs from the supernatant, respectively. The method described has been tested with artificial mixtures containing LPSs, polysaccharide, protein and RNA and subsequently employed for the preparative purification of two LPSs of different origin, namely the extracellular LPS secreted by *Escherichia coli* E49 into the culture medium, and the cell wall bound LPS from *Pseudomonas aeruginosa* VA11465/1. Compared to currently used methods for LPS purification such as enzymatic digestion and ultracentrifugation, the chromatographic separation reported here combines superior purity with minimal loss of LPS, high reproducibility and simple handling. The removal of contaminants such as protein, RNA and polysaccharides and the recovery of LPSs were monitored by appropriate assays.

Keywords: Cell wall composition; Lipopolysaccharides

1. Introduction

Lipopolysaccharides (LPSs) are an integral part of the outer cell wall of Gram-negative bacteria and play an important role in the interaction of the microorganism with its environment [1]. Upon infection with Gram-negative bacteria, LPSs are responsible for a number of pathological reactions in the human body, such as fever, diarrhoea, blood pressure drop, septic shock and death [2–4]. In addition, they also trigger various physiological reactions such as interferon secretion, production of colony stimulating factors and other cytokines [4–6]. Based on their LPS structure, Gram-negative species are divided into various serological strains. LPSs of smooth strains consist of a lipophilic portion termed lipid A which serves as anchor for the molecule in the outer membrane, a core region and the so-called O-specific side chain which is an outer polysaccharide portion consisting of repeating oligosaccharide units [7]. Lipid A causes the endotoxin effects, whereas the O-specific side chain of LPS is mainly responsible for the antigenic properties of the macromolecule [8]. LPS of rough mutants contains no

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O-specific side chain and thus consists only of the core region.

Investigation of LPS structure and their interactions with mammalian cells requires the preparation of significant amounts of highly purified material. Classical methods which are still used as routine methods in many laboratories are the so-called hot phenol–water extraction [9] for smooth LPSs and the phenol–chloroform–petroleum ether extraction [10] for rough LPSs. However, contaminants such as proteins, RNA and polysaccharides are also being extracted, and the preparation of suitably pure LPS requires additional purification steps by ultracentrifugation [11], enzymatic digestion of macromolecular contaminants with subsequent removal of breakdown products by dialysis [12] and chromatographic methods like affinity chromatography [13] or gel filtration [14,15]. Ultracentrifugation suffers from a low recovery for LPSs and provides only sub-optimal purity of the product. Polymeric contaminants such as RNA and polysaccharides can be removed by enzymatic digestion. However, the enzymes have to be removed in turn by another hot phenol–water extraction. Removal is generally not quantitative and results in a significant loss of LPSs.

A few years ago, a method for purification of LPSs from *Escherichia coli* and *Salmonella* sp. by means of hydrophobic interaction chromatography (HIC) with Octyl Sepharose was published. The procedure was reported to provide highly purified LPSs from hot phenol–water extracts or from culture supernatants [16], although no data were given with respect to yield and purity attained. In our hands, this protocol did not provide satisfactory results for the isolation of LPSs from the supernatant of *Escherichia coli* (*E. coli*) E49. The retention of *E. coli* E49-LPS on the sorbent was not sufficiently strong to separate LPSs from accompanying contaminants. We tested eluents over a wide range of hydrophobic strength, but no significant retention of LPSs could be achieved even with eluents containing only a very small proportion of organic solvent (*n*-PrOH). Since we needed sizable quantities of material for structural analysis and biosynthetic investigations, a modified HIC method for the rapid and reliable purification of LPSs of different origin was developed and validated.

2. Experimental

2.1. Materials

All chemicals used were of analytical or biochemical grade. HPLC grade *n*-PrOH (Roth, Karlsruhe, Germany) and water, purified over a mixed-bed water demineraliser Seradest SD 2800 (Seral, Ransbach-Baumbach, Germany) were used for chromatography. Unless stated otherwise, chemicals and solvents were purchased from Sigma (Deisenhofen, Germany).

2.2. Equipment

Fermentations were carried out in a 5-l Biostat B laboratory fermenter (B. Braun Biotech, Melsungen, Germany). Ultracentrifugation was performed with a Beckman L7-35 (Beckman, Palo Alto, CA, USA) ultrasonication with a Labsonic U (B. Braun Biotech). Ultrafiltration of LPSs was carried out with a Pellicon-System (Millipore, Bedford, IN, USA) over an Omega membrane MWCO (molecular-mass cut-off) 10 k (Filtron, Karlstein, Germany). For determination of protein and polysaccharide content, microtitre plates were read on a Microplate Reader 3550-UV with Microplate Manager 4.0 (Bio-Rad, Hercules, CA, USA). A HTS 7000 Bio Assay Reader with HTSoft 1.0 (PE Biosystems, Norwalk, CT, USA) was used for LAL tests. For HIC, a Bio-Rad Econo-System and glass columns with water jacket (Krannich, Göttingen, Germany) were used. UV–Vis measurements were carried out with a DU 640 spectrophotometer (Beckman, Palo Alto, CA, USA).

2.3. Bacterial strains and culture

E. coli strain E49 was a clinical isolate obtained from the Medical School of the Friedrich-Schiller-University, Jena and was stored in the Microbank system (Mast-Diagnostika, Reinfeld, Germany) in liquid N_2. Starting from liquid precultures, bacteria were grown aerobically at 37°C in a laboratory fermenter in a minimal medium containing 2.0 g KH_2PO_4, 9.6 g K_2HPO_4, 0.5 g Na-citrate, 0.1 g $MgSO_4 \cdot 6H_2O$, 20.0 g $(NH_4)_2SO_4$, 4.0 g glucose and 100 ml of dialysed yeast extract per litre (pH 7.0).

The yeast extract was prepared by dialysing a solution of 10 g yeast extract (Roth, Karlsruhe, Germany) in 100 ml distilled water for 24 h against 1 l distilled water (exclusion limit 10 000 Da). Upon reaching the late logarithmic growth phase, culture was stopped by centrifugation (11 000 *g*, 4°C, 20 min).

Pseudomonas aeruginosa VA11465/1 was isolated in 1998 from an clinical skin infection in the Medical School of Friedrich-Schiller-University, Jena and stored in the Microbank system in liquid N_2. Starting from liquid precultures, the cells were grown aerobically at 37°C in a laboratory fermenter in modified LB medium (10 g tryptone, 5 g yeast extract, 2 g glucose per litre). Cultures were harvested in the late log phase by centrifugation (11 000 *g*, 4°C, 20 min). Cell pellets were washed (once with 20 mM Na_2-EDTA in 0.85% NaCl, twice with 10 mM $MgSO_4$, twice with water) to remove loosely bound extracellular polysaccharides.

2.4. Preparation of LPSs

Supernatant of *E. coli* E49-cultures was freed from low-molecular-mass substances and concentrated up to 10% (v/v) of the starting volume by ultrafiltration. Crude LPS was obtained by lyophilisation. Defined starting solutions were prepared by ultrasonic treatment.

Cell-wall associated LPS from *P. aeruginosa* VA 11465/1 was prepared using the hot phenol–water method [9]. Briefly, 30 g of wet cell mass were thoroughly suspended in 100 ml distilled water, warmed to 68°C and mixed with one volume of prewarmed (68°C) phenol containing 10% (v/v) water. The mixture was stirred for 15 min at 68°C and cooled in an ice bath to 2°C. After centrifugation (6000 *g*, 4°C, 15 min) the upper water phase was siphoned off and dialysed for 72 h against distilled water. Crude LPS was recovered by lyophilisation.

2.5. Column liquid chromatography

HIC was carried out on Butyl Sepharose 4 Fast Flow (Pharmacia, Uppsala, Sweden). For exploratory purposes, a small column (20×1 cm I.D.; length of chromatographic bed 15 cm) was employed. Preparative purification was achieved on a larger column (30×3 cm I.D.; chromatographic bed 25 cm). The columns were thermostatted at 20°C.

The mobile phase consisted of sodium acetate buffer (0.2 *M*, pH 4.7) and *n*-PrOH. The eluent programme was as follows: sodium acetate buffer 100% over 4 min for analytical and 9 min for preparative columns, followed by a linear gradient to sodium acetate buffer–*n*-PrOH (60:40, v/v) over 36 and 90 min, respectively. The flow-rates were 2.5 ml min^{-1} and 12 ml min^{-1}, corresponding to a linear velocity of 191 cm h^{-1} (analytical column) and 102 cm h^{-1} (preparative column). Injection volume was 200 μl on analytical column and 5 ml on the preparative column, corresponding to a sample amount of 2 mg and 50 mg crude LPS. Fraction sizes were 5 ml and 25 ml, respectively.

2.6. Ultracentrifugation

Ultracentrifugation (100 000 *g*, 4 h) was carried out at 4°C with a LPS stock solution (5 ml; 12.17 mg ml^{-1}).

2.7. Enzymatic RNA digestion

Freeze dried crude LPS was treated with an RNase incubation solution (1 ml RNase solution per 10 mg of crude LPS) for 12 h at 37°C. RNase solution contained 0.2 mg ml^{-1} RNase from bovine pancreas (70 units mg^{-1}; Serva, Heidelberg, Germany) in Tris–HCl buffer (pH 7.5). RNase was removed by hot phenol–water extraction [9] and subsequent dialysis.

2.8. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out according to Laemmli [17]. Reagents used were from a sodium dodecyl sulphate (SDS)–PAGE set (Biometra, Göttingen, Germany; product No. FL-P 10). The gel system consisted of a stacking gel (3% *N,N′*-bis-methylene acrylamide) and a separating gel (12.5% *N,N′*-bis-methylene acrylamide). To 20 μl of sample (about 1 mg ml^{-1} LPS), 20 μl sample buffer was added and heated for 7 min in a boiling water bath. After cooling to room

temperature, 4 μl dithioerythritol were added, and 7 μl of the mixture applied on every gel slot. Electrophoresis was carried out on a Bio-Rad power supply (Model 1000/500) with 10 min at 10 mA, then 20 mA with variable voltage. Samples were running from cathode to anode. Staining of gels with alkaline silver nitrate was performed according to Tsai and Frasch [18].

2.9. Analytical methods

To ensure the reproducibility of results, all experiments were performed repeatedly at minimum in triplicate. For quantitative data, measurements were carried out in triplicate.

Protein content was determined according to the method of Bradford [19]. A protein assay dye concentrate (Bio-Rad; Cat. No. 500-0006) was used, and the assay was carried out in 96-well microtitre plates. Absorption was measured at 595 nm. Bovine albumin (Albumin Fraktion V; Merck, Darmstadt, Germany; Cat. No. 1.12018.0025) was used as reference, and water as blank. Detection limit was 5 $\mu g\ ml^{-1}$. It was found that the reagent produced a weak turbidity in highly concentrated LPS solutions. This turbidity is likely the result of a hydrolytic release of the lipid A portion of LPS due to the high concentration of phosphoric acid in the protein dye reagent.

RNA content was determined by measurement of UV absorption at 256 nm. RNA from yeast (Boehringer Mannheim, Mannheim, Germany) was used as reference, and water served as blank. The detection limit of this test was 5 $\mu g\ ml^{-1}$.

Polysaccharide content was determined by phenol–sulphuric acid reagent [20] in a microtitre plate assay. Absorption was measured at 490 nm, with water as blank and dextran 150 (Pharmacia) as standard. The detection limit of the polysaccharide determination was 20 $\mu g\ ml^{-1}$.

LPS was determined with a commercial LAL kit (Kinetic-QCL; Bio Whittaker, Walkersville, MD, USA; Cat. No. 50-650U) which allows quantitative determination of LPSs in references with a known endotoxin standard (*E. coli* O55:B5 endotoxin). The assay was carried out according to the protocol of the manufacturer. The detection limit of this LAL test is indicated to be 0.005 EU ml^{-1} (1 pg ml^{-1}).

3. Results

Currently used methods for LPS purification take advantage of differences between LPSs and contaminants in properties such as partition coefficient, sedimentation constant or stability towards enzymatic digestion. HIC, on the other hand, is based on the interaction of a hydrophobic matrix with the lipophilic moiety of the analyte. Based on the structural differences between LPSs and contaminants, a significantly stronger interaction could be anticipated for the former. Attempts to employ the conditions used by Fischer [16] failed, the extracellular LPS from *E. coli* E49 being poorly retained on Octyl Sepharose. Decreasing the hydrophobic strength of the eluent by lowering the proportion of organic solvent did not provide any major improvement. Given this unsatisfactory experience with Octyl Sepharose, we decided to test Butyl Sepharose 4 Fast Flow.

Development of the method was carried out with LPS from *E. coli* E49. From previous investigations, this particular strain was known to release significant amounts of LPS into the culture medium. An enriched LPS fraction could be obtained from the supernatant by ultrafiltration without need for prior hot phenol–water extraction. The crude LPS fraction thus obtained was devoid of low-molecular-mass contaminants but still contained RNA, protein and polysaccharides [21]. For method development and validation, this LPS preparation was spiked with equal amounts of RNA, protein and polysaccharides. The test mixture containing 2 mg ml^{-1} each of LPS, bovine serum albumin, yeast RNA and dextran 150 was separated on an analytical Butyl Sepharose column using a sodium acetate–*n*-PrOH gradient (for details see Experimental). Excellent separation of LPS from the contaminants was achieved. Monitoring of the separation and purity of the LPS fraction are shown in Figs. 1 and 2 and Table 1. Analysis of the individual fractions for LPS, protein, RNA and polysaccharide content (Fig. 1) showed that the contaminants were eluted with aqueous buffer. Due to their hydrophilic properties, RNA and polysaccharides do not interact with the stationary phase, and the ionic strength of the buffer also precludes significant retention of proteins. LPS was eluted with 40% *n*-PrOH. LPS-containing fractions

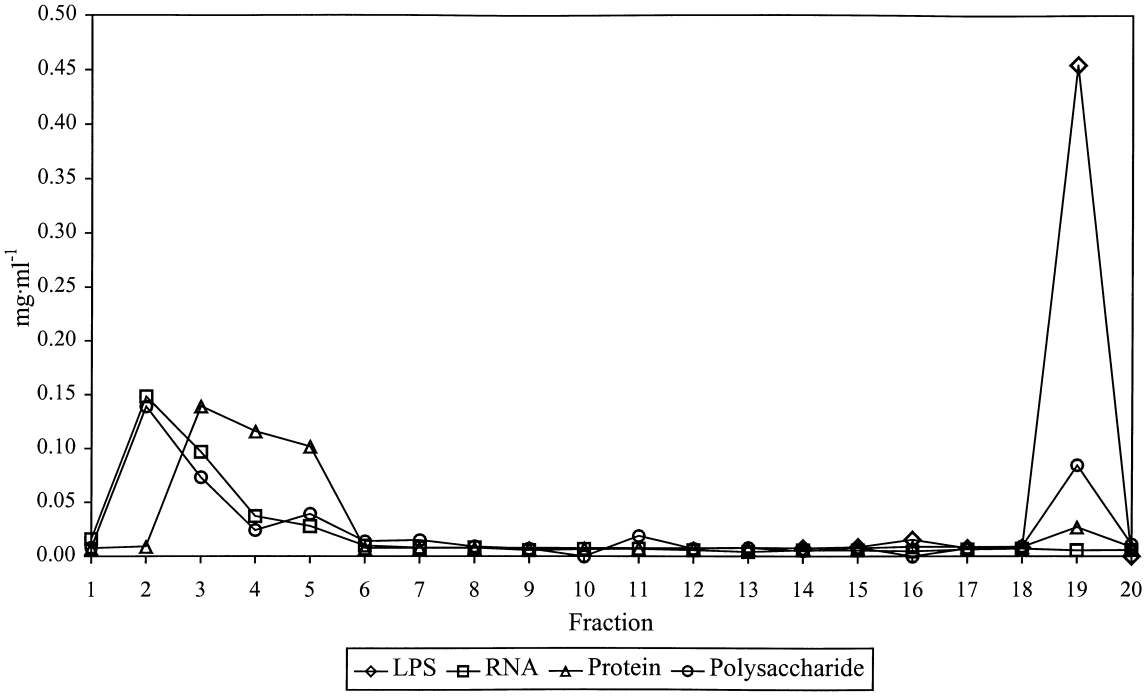

Fig. 1. Elution profile of *E. coli* E49-LPS and contaminants on analytical Butyl Sepharose column (20×1 cm I.D.). For further conditions, see Experimental. The polysaccharide peak in fraction 19 is due to the polysaccharide portion of LPS (O-specific chain). The ''protein peak'' in the same fraction is an artifact (see Experimental and Results).

were detected by the highly sensitive and specific LAL assay. The ''protein peak'' appearing in the LPS-containing fractions (see Fig. 1) is an artifact resulting from hydrolytic release of lipid A under the strongly acidic conditions of the protein assay. The absence of protein was confirmed by UV–Vis spectroscopic analysis. The high purity of the LPS fraction was confirmed by PAGE (Fig. 2). The typical ladder-like pattern of LPS is due to the varying number of repeating oligosaccharide moieties of the O-specific side chain. In the LPS fractions, RNA, protein and polysaccharides were below the detection limit of the respective assays. The recovery for LPS was 95% (m/m) with respect to the LPS quantity injected. Repeated preparative purification of crude E49 LPS gave comparable results and confirmed the good reproducibility of the method.

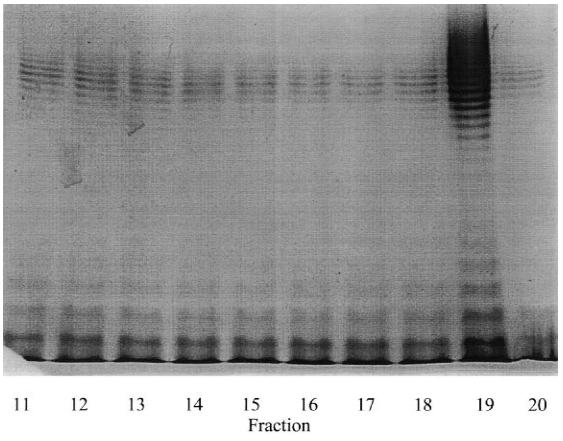

Fig. 2. Purity check of *E. coli* E49-LPS obtained by analytical HIC. PAGE of fractions 11–20 (silver staining). For details, see Experimental.

In order to test the wider applicability of our method, cell wall bound LPS from *P. aeruginosa* VA11465/1 was submitted to an identical purification protocol. Crude LPS was obtained from the bacterial cell wall by hot phenol–water extraction. This LPS fraction contained small amounts of pro-

Table 1
Comparison of various methods for purification of LPS, with respect to purity achieved and percentage of recovery of LPS and accompanying biopolymers (for details, see Experimental)

	HIC		Ultracentrifugation		RNA digestion	
	E. coli E49	*P. aeruginosa* VA 11465/1	*E. coli* E49	*P. aeruginosa* VA 11465/1	*E. coli* E49	*P. aeruginosa* VA 11465/1
Purity (%)	99[a]	99[a]	25	88	25	87
Recovery in LPS fraction (%)						
LPS	95	100	25	5	50	100
RNA	0	0	23	0.6	50	21
Protein	0	0	41	0	50	22
Polysaccharides	0	n.d.[b]	15	n.d.[b]	100	n.d.[b]
Time required for preparation of 50 mg LPS (h)	12[c]	12	12	12	80	80

[a] All contaminants below detection limit.
[b] Not contained in crude LPS and therefore not determined.
[c] Including dialysis and lyophilisation.

tein and a significant proportion of RNA, but were devoid of accompanying polysaccharides. Results from a preparative separation are shown in Figs. 3 and 4 as well as in Table 1. Again, the protein peak in the LPS fraction was found to be an artifact caused by the protein assay reagent in presence of LPS. The recovery of LPS was virtually 100% (m/m). Noteworthy is the high proportion of RNA (50–55%, m/m) which could be removed from the crude LPS preparation. RNA content was below the de-

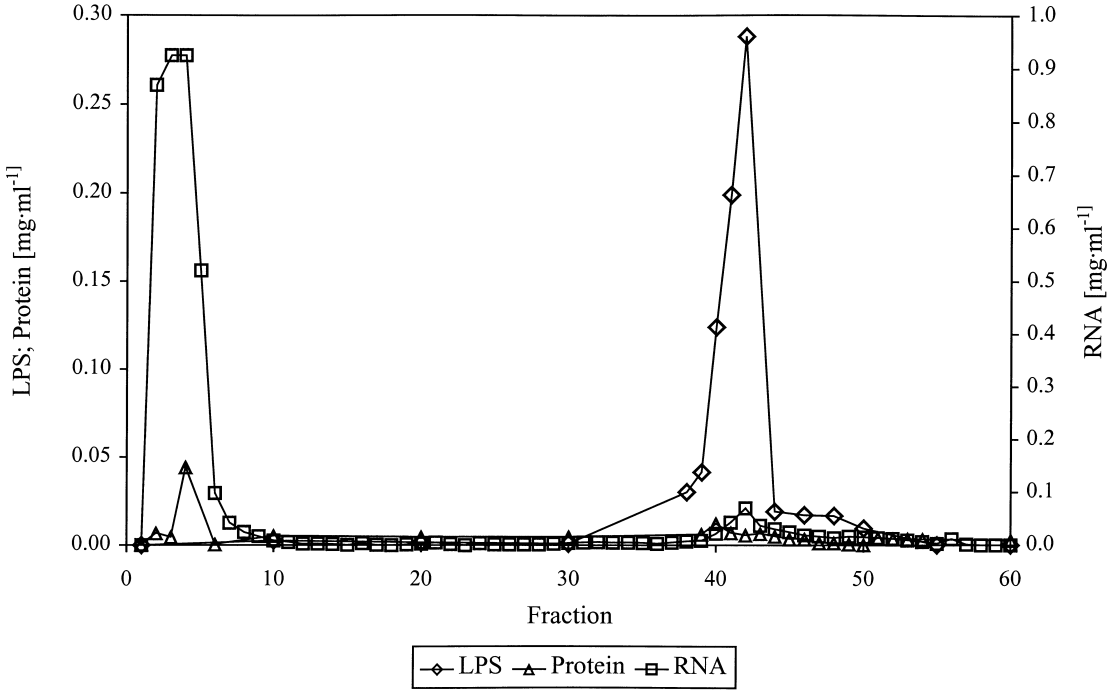

Fig. 3. Elution profile of *P. aeruginosa* VA11465/1-LPS and contaminants on preparative Butyl Sepharose column (30×3 cm I.D.). For further conditions, see Experimental. The ''protein peak'' in the LPS fraction is an experimental artifact (see Experimental and Results).

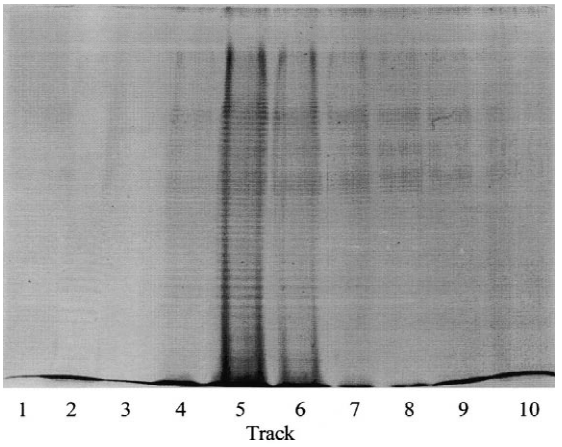

Fig. 4. Purity check of *P. aeruginosa* 11465/1-LPS obtained by preparative HIC. PAGE of crude LPS (track 2) and HIC-fractions 39–45 (tracks 4–10) after silver staining. For details, see Experimental.

tection limit – after purification on Butyl Sepharose. The results obtained with the cell wall bound LPS of *P. aeruginosa* VA11465/1 confirm our findings with extracellular LPS from *E. coli* E49.

No data are available, to the best of our knowledge, on the efficiency and recovery achievable with other LPS purification methods, and we therefore compared ultracentrifugation and RNA digestion with HIC. For experiments with *E. coli* E49 LPS, the same test mixture as for the HIC experiments was used, whereas LPS from *P. aeruginosa* VA11465/1 was obtained by hot phenol–water extraction.

After ultracentrifugation, LPS, protein, RNA and polysaccharide contents in the supernatant and pellet were determined. The data in Table 1 reveal that no enrichment of LPS could be achieved by this method, despite of a significant loss of LPS. RNase treatment is frequently employed for the purification of LPS fractions with a high RNA content. Enzymatic digestion of the spiked E49 LPS led to a reduction of the RNA by 50%. However, the increased purity of the LPS was offset by a recovery of only 50% which is due to the phenol–water extraction required for removal of the RNase.

With the protocol described, up to four separations a day of 50 mg crude LPS each could be managed with the preparative column employed. Considering a RNA content of 52.5% and 3% of protein, this corresponds to 90 mg purified LPS which can be obtained as a lyophilized powder within 30 h under mild conditions. The robustness of the purification method is documented by the fact that we have used the preparative Butyl Sepharose column for more than 80 separations without any noticeable loss in performance.

4. Discussion

Bacterial lipopolysaccharides are essential components in the cell wall of Gram-negative bacteria, where they co-occur with a multitude of other macromolecules. Thanks to the lipid A portion, the LPS is anchored in the cell wall. Nonetheless, a growing number of bacterial strains have been shown to release LPS into their surroundings. Extracellular LPS co-occurs in the culture medium with bacterial polysaccharides and proteins, whereas extracts of cell wall bound LPS typically contain RNA and proteins. Currently used extraction methods allow for almost complete removal of proteins. Polysaccharides and RNA, however, cannot be removed by classical phenol–hot water extraction and thus significantly contaminate crude LPS preparations.

Preparation of highly purified LPS is a necessary prerequisite in various areas of LPS research. The ultrastructures formed by LPS self-association, for example, may be significantly altered in the presence of other macromolecules. It is therefore surprising how little attention has been paid in the literature to the purity assessment of LPS used for such investigations. To the best of our knowledge, no comparative data have been published on the purity of LPS that can be obtained by the various purification protocols. Our findings indicate that ultracentrifugation and RNA digestion cannot be considered as effective means for purification. In our comparison of purification methods, we did not evaluate affinity chromatography and gel filtration. These methods have been sporadically used for LPS purification [22] and may, in principle, be effective means for obtention of high purity LPS. The lack of a wider applicability, however, severely restricts their usefulness. In contrast to all the methods mentioned above, purification on Butyl Sepharose is an efficient, rapid,

robust method of general applicability and therefore a superior alternative to classical methods.

The chromatographic behaviour of LPS on Octyl- and Butyl Sepharose was quite unexpected. Due to the higher hydrophobicity of the octyl residues, one would assume stronger retention of LPS on Octyl Sepharose. The opposite, however, was observed with respect to our LPS samples. A reasonable explanation for this behaviour may be the difference in ligand density of the two sorbents. The ligand density in Butyl-Sepharose is significantly higher (50 μmol ml^{-1} gel vs. 5 μmol ml^{-1} gel in Octyl Sepharose) [23], and the higher degree of substitution apparently leads to significantly stronger hydrophobic interaction with the solutes than in case of Octyl Sepharose.

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