

Purification of *E. coli* 055:B5 lipopolysaccharides by size exclusion chromatography

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ABSTRACT

Several methods for the extraction of endotoxins or lipopolysaccharides from Gram-negative bacteria have been described. However, the product is often contaminated with nucleic acids or proteins in variable proportions depending on the extraction method used. Molecular and immunological studies require further purification of the raw LPS. Here we present a simple method for the purification of raw LPS obtained by the standard hot phenol-water procedure using size exclusion chromatography in Sepharose CL-6B. We demonstrated that the DNase and RNase treatment of the sample is necessary before the chromatographic step to abrogate nucleic acid contamination in the LPS fraction. The spectrophotometric properties of pure LPS were assessed, supporting the usefulness of on-line absorbance at 206 nm to monitor the elution of LPS and oligonucleotides. The mobile phase used (0.2 M NaCl) does not absorb at 206 nm while it promotes LPS aggregation and therefore allows its separation from oligoribonucleotides and oligodeoxyribonucleotides. The yield of pure LPS was of about 98%. Chemical and biological characterizations were conducted to assess the feasibility of the procedure.

Key words: endotoxin, lipopolysaccharide, purification, chromatography

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RESUMEN

Purificación de lipopolisacáridos de *E. coli* 055:B5 por cromatografía de exclusión molecular. Se han descrito varios métodos para la extracción de endotoxinas o lipopolisacáridos (LPS) de bacterias gramnegativas; sin embargo, el producto suele estar contaminado con ácidos nucleicos y/o proteínas en una proporción que depende del método de extracción empleado. Para estudios moleculares e inmunológicos, se requiere la purificación adicional del extracto de lipopolisacárido. En este artículo se describe el desarrollo de un método para la purificación de un extracto de LPS, obtenido por el método de Westphal, utilizando cromatografía de exclusión molecular en Sepharosa CL-6B. Se demostró que es necesario el tratamiento de la muestra con ADNasa y ARNasa antes de la cromatografía, para obtener la fracción correspondiente a los LPS libres de ácidos nucleicos. Se evaluaron las propiedades espectrofotométricas del LPS en el ultravioleta lejano y se confirmó que es posible la detección inmediata a 206 nm de las fracciones de interés (LPS y ácidos nucleicos), sin emplear métodos químicos más engorrosos. Como fase móvil, se empleó el NaCl 0.2 M, el cual no absorbe a 206 nm y conserva los agregados micelares de los LPS. Ello contribuye a la separación entre la fracción lipopolisacáridica y los oligonucleótidos. Con este procedimiento se obtuvo un LPS con aproximadamente 98% de pureza. Se realizaron controles químicos y biológicos para la evaluación del proceso.

Palabras claves: endotoxinas, lipopolisacáridos, purificación, cromatografía

Introduction

Endotoxins or lipopolysaccharides (LPS) from Gram negative bacteria can be extracted by several methods such as: trichloroacetic acid extraction at 4 °C [1], aqueous butanol [2], triton/Mg²⁺ [3], cold ethanol [4] and extraction in water at 100 °C [5]. Other extraction methods with phenol, chloroform, petroleum-ether [6] and methanol [7] have been described specifically for rough LPS (R-LPS).

The most frequently employed method is that of Westphal [8], also known as the hot phenol extraction procedure. Its main advantage is the high yield in LPS, free from contaminant proteins [2, 8]. Ultracentrifugation has been proposed to eliminate those contaminants [8-10]; however, sedimented lipopolysaccharides usually contain a certain amount of nucleic acids, while an important percentage of LPS is lost in the nucleic acid rich supernatant [8].

Size exclusion chromatography seems to be the method of choice to purify R-LPS or lipooligosaccharides (LOS). A 50% increase in yield has been described in the size

exclusion chromatography purification of *N. meningitidis*, *H. influenzae* and *B. pertussis* LOS compared to ultracentrifugation [11]. Even FPLC has been employed for the purification of *N. meningitidis* LOS [12].

LPS from smooth bacterial strains, which contain long O side chains, have also been purified previously by size exclusion chromatography [2]. However, the main disadvantage of this chromatographic method applied to LPS purification is the difficulty to detect LPS containing fractions. Several chemical methods have been used for this such as the quantitative determination of colitose (3.6 dideoxy-L-galactose) [2], 2-keto-3-deoxyoctulosonic acid (KDO) [11] and total hexoses [13]. Those methods are cumbersome and time consuming.

In the present study we assessed the spectroscopic properties of LPS in the far ultraviolet (UV) spectrum, to detect relevant fractions in the purification process at 206 nm. On the other hand, treating the samples initially with nucleases seems to be a viable option to

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improve the separation of LPS and nucleic acids. This procedure has been used before in either ultracentrifugation [14] or chroma-tographic methods [11-12].

Ribonucleic acid (RNA) is the main contaminant (50% to 60%) of the LPS extracts obtained by the Westphal method [8], therefore, some protocols include an RNA elimination step, with RNase [2, 10, 12] or cetavlon (cetyltrimethylammonium bromide) [8]. However, procedures using DNase and RNase treatment before the main purification step have also been followed to separate LPS from DNA and RNA respectively. [11, 13, 15]. Therefore, here we define the need of a nuclease treatment to obtain an LPS chromatographic fraction free from nucleic acids and to evaluate if the RNase treatment alone is required or both molecules should be hydrolyzed to obtain an LPS with a higher degree of purity. Thus, this paper describes a procedure that includes size exclusion chromatography for the purification of crude *E. coli* O55:B5 LPS obtained by the Westphal method.

Materials and methods

LPS Extraction

LPS were extracted according to the Westphal and Jann method [8] with minor modifications

The bacterial suspension (5% dry biomass in 100 mL), was mixed with the same volume of 90% phenol previously heated at 67 °C, and incubated at the same temperature in an orbital shaker at 160 rpm for 15 min. The mixture was then placed on ice to facilitate the separation in phases and centrifuged for 20 min at 5000 g. The aqueous phase was collected. A second extraction was made on the mixture of phenol and the cellular pellet by adding 100 mL of distilled water at 67 °C. Both aqueous phases were combined and dialyzed against distilled water until the phenol was completely eliminated (0 absorbance at 260 nm in the water outside the dialysis tube). The sample was then clarified by centrifugation at 10000 g for 20 min at room temperature to eliminate the insoluble material. LPS were concentrated by alcohol precipitation as follows: Sodium acetate was added at a final concentration of 0.15 M, the tube was placed on ice and cold 96% ethanol was added drop by drop for a final sample/ethanol proportion of 1:4. The mixture was incubated for 24 hours at -20 °C. The pellet was then collected by centrifugation at 4000 g, suspended at 25 mg/mL in distilled water and lyophilized.

Nuclease treatment of samples before the chromatographic step

Two procedures were compared to evaluate the efficacy of the nuclease treatment before chromatography in the nucleic acid content of the final product. In the first procedure the lyophilized LPS extract was dissolved at 25 mg/mL in 0.2 M NaCl (final volume of 10 mL), and incubated for 1 h at 37 °C with RNase at 0.5 mg/mL. In the second procedure both RNase and DNase were used at 0.5 mg/mL and 50 µg/mL respectively, following the same methodology. In both cases the enzymatic hydrolysis was stopped by incubating the mixture at 65 °C for 12 min [15]. A sample without the nuclease treatment was used as the negative control. In all cases the sample was homogenized by vigorous stirring in a

vortex for 5 min, or by sonication in a Branson 1200 ultrasonic bath, and filtered using 0.45 µm cellulose acetate filters.

Liquid chromatography

LPS was purified with a low-pressure chromatographic system Gradifrac™ (Pharmacia). LPS extracts (8 to 10 mg/mL in 0.2 M NaCl) were loaded in an XK 16/70 column (Pharmacia) packed with Sepharose CL- 6B (Pharmacia), and equilibrated with 0.2 M NaCl. Separation was performed at 9 cm h⁻¹ flow rate and absorption was monitored at 206 nm. Fractions of 3 or 5 mL were collected for the analysis. The available distribution coefficient (K_{av}) was used to characterize the elution volume of the samples. Fractions corresponding to the LPS peak were mixed and concentrated by alcohol precipitation as described above but without adding salt. The pellet was suspended in distilled water and lyophilized.

Chemical analysis

The qualitative detection of 2-keto-3-deoxyoctulosonic acid (KDO) by the thiobarbituric acid (TBA) micro-method, described by Karkhanis [16], was performed to identify LPS containing fractions. The presence of nucleic acids was determined by a spectrophotometric scan from 200 to 300 nm. Nucleic acid contents in LPS extracts or chromatographic fractions were estimated by assuming that a 40 µg/mL RNA solution gives 1 unit of absorbance at 260 nm [12].

Contaminant proteins were measured by the Bradford method, using bovine serum albumin as the standard, following the procedure described by Bollag et al [17]. LPS were chemically analyzed by SDS-PAGE in 15% polyacrylamide gels [18]. The gels were developed by the LPS specific silver staining method using the methodology described by Hancock and Poxton [19]. The potency of LPS was determined by the limulus amoebocyte assay gel-clot method (LAL) (Biowhitaker Inc.), which has a sensitivity of 0.06 endotoxin units per milliliter (UE/mL), using a previously standardized protocol [20]. To study the relationship between optical density at the far UV spectrum and LPS concentration, pure LPS were dissolved in 0.2 M NaCl at concentrations of 1 µg/mL to 2 mg/mL and absorbance was measured in a Genesys 2PC spectrophotometer against 0.2 M NaCl as the background control.

Results

The yield of LPS in the extracts in relation to the dry bacterial biomass was 8%. The potency of the extract was 4.8 endotoxin units (EU/ng) measured by LAL. This value is similar to those reported for LPS extracts from the same bacterial strain [21]. The nucleic acid content was of 44% (table 1). KDO qualitative detection gave absorbance values of 0.246 UA and 0.003 UA for peaks one and two respectively, which suggests that most LPS molecules elute in the first chromatographic peak, while most nucleic acids elute in the second peak.

Figure 1 shows that the resolution is not optimal when the LPS extract is applied directly in the chromatographic process. K_{av} values of 0 and 0.57 were measured for LPS and nucleic acid fractions respectively.

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Table 1. Control of nucleic acid and proteins in lyophilized LPS extracts (A), LPS obtained by chromatographic procedures without previous nuclease treatment (B) LPS purified after hydrolysis with RNase and DNase (C)

	A	B	C
Nucleic acid	44 ± 0.5292 %	12 ± 0.3605 %	1.75 ± 0.3258 %
Proteins	<1 %	<1 %	<1 %

LPS solution was prepared in triplicate for nucleic acid and protein determination. Standard deviation values are shown.

The spectrophotometric scanner of the lipopolysaccharide fraction shows a maximum absorption at 260 nm (Figure 2), which suggests the presence of contaminant nucleic acid.

Figure 3 shows the chromatographic profile of the sample previously treated with RNase. A better resolution is achieved in this case with Kav values of 0 and 0.84 for fractions one and two, respectively.

However, from a qualitative point of view, contaminant nucleic acids, presumably DNA, can still be detected (maximum OD 260 nm = 0.253 UA).

When nucleic acids are hydrolyzed before chromatography, the maximum absorption at 260 nm disappears in the lipopolysaccharide fraction (Figure 4), which indicates a drastic reduction in the level of contaminant DNA. Under these conditions LPS shows a maximum absorption at 204 nm and no absorption at 260 to 280 nm, the absorption range for nucleic acid and proteins.

The differences between the scanning profile of LPS and oligonucleotides were easily distinguished. In this case the resolution of the process was similar to the previous experiment where samples were treated with RNase alone. Kav values were of 0 and 0.87 for signals one and two respectively.

Table 1 summarizes the nucleic acid and protein contents for the different stages of the purification process.

Electrophoretic analysis (Figure 5) showed the same band pattern in pure LPS and raw LPS. The potency of purified LPS was 48.4 UE/ng, measured by LAL.

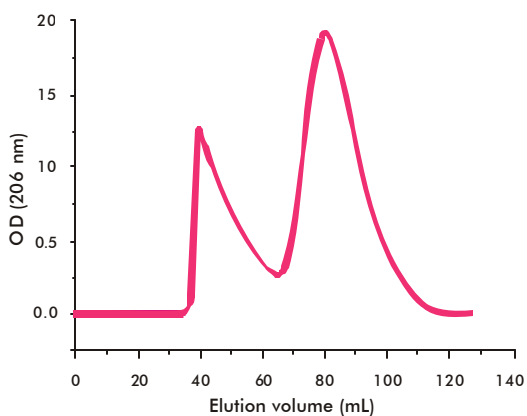


Figure 1. Chromatographic profile of a crude LPS sample loaded without previous nuclease treatment (control). Flow rate of 9 cm h⁻¹, injection volume, 2 mL of LPS extract at 5 mg/mL.

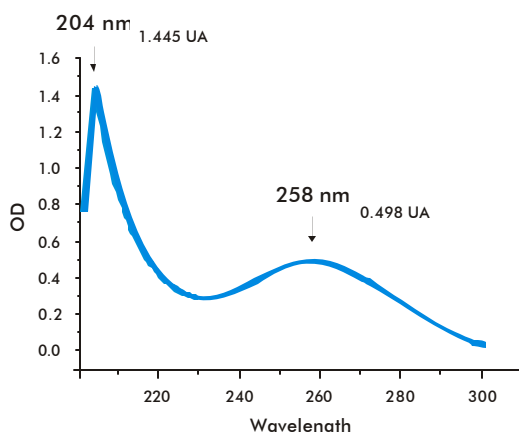


Figure 2. Spectrophotometric scanner from 200 to 300 nm of pooled lipopolysaccharide fractions obtained in the chromatographic process without previous nuclease treatment.

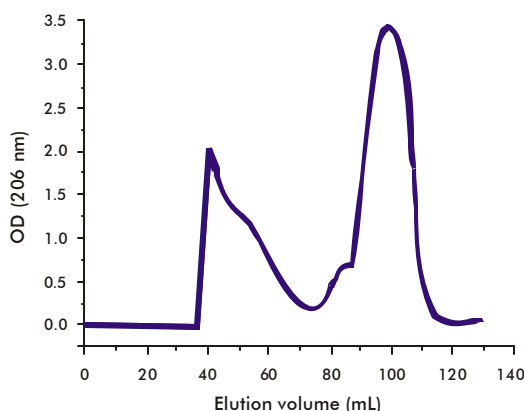


Figure 3. Chromatographic profile of LPS sample applied after previous RNase treatment. Injection volume 2 mL at 8 mg/mL, flow rate 9 cm h⁻¹.

The ultrasonic treatment or vortex did not disaggregate the LPS. However, the application of either method is needed to homogenize the sample, and to facilitate microfiltration (using 0.45 or 0.2 µm membranes) before applying the chromatographic process. Hydrolysing the sample (at 25 mg/mL) by the nuclease treatment greatly reduces viscosity. The relationship between absorption at 206 nm and pure LPS concentration follows a linear pattern (R² 0.99) for concentrations of 1 µg/mL to 1 mg/mL (Figure 6). At higher LPS concentrations, optical density still increases but linearity is lost. The adsorption values at 200 nm shows a linear correlation of 1 µg/mL to 0.5mg/mL (Figure 6).

Discussion

While raw LPS preparations are useful for the electrophoretic analysis, additional purification is required for molecular and immunological studies [12]. Although it is possible to separate the main LPS and nucleic acid fractions when crude extracts are applied directly to the chromatographic columns, contaminant

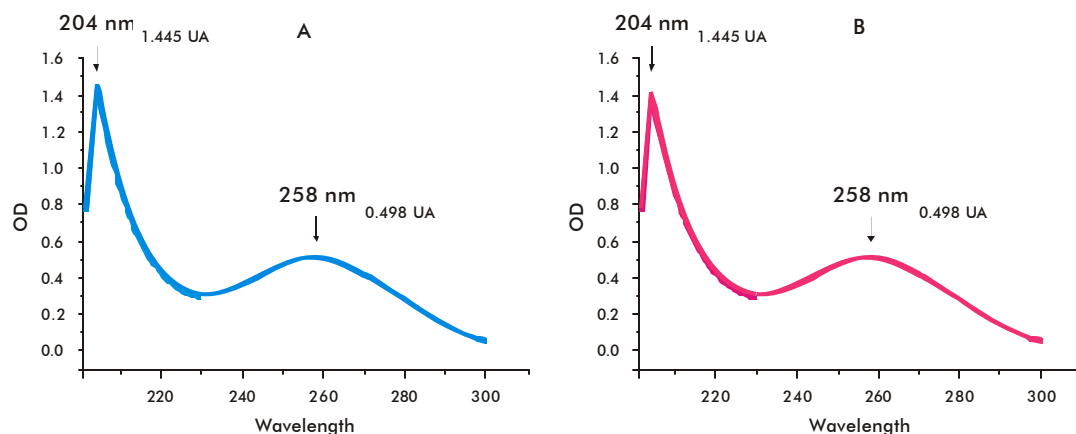


Figure 4. Spectrophotometric scanner from 200 to 300 nm of pooled lipopolysaccharide fractions obtained in the chromatographic process after previous RNase y DNase treatment A). Chromatographic fractions corresponding to the first peak (LPS). B) Second peak (oligonucleotides).

nucleic acids (12%) still remains in the final LPS preparation.

RNA is the main contaminant in LPS extracts obtained by the Westphal method [8]; the RNase mediated hydrolysis of the sample does not eliminate all contaminating nucleic acids, although an improvement in resolution is observed. Therefore, even if RNA is the main contaminant, an additional DNase treatment is required. By treating the sample with both nucleases before chromatographic separation, 98% of the pure LPS is recovered.

The hydrolysis of nucleic acid polymers to oligoribonucleotides and oligodeoxyribonucleotides cooperates with the mobile phase to increase the resolution of the process while leading to a reduction in sample viscosity. This is one of the aspects limiting

the concentration of the sample before size exclusion chromatography. The choice of a mobile phase is essential in establishing a chromatographic method for the purification of any macromolecule.

Detergents (sodium deoxycolate) and EDTA in the mobile phase are used to purify LPS by chromatographic methods [11-12]. The combination of these compounds offer a strong disaggregating effect, yielding LPS monomers of approximately 20 kDa [22]. This effect gives higher elution volumes for LPS containing fractions. This could be responsible for the findings of Wu et al. [11], where the DNase mediated hydrolysis of LPS extracts before chromatography increased the level of protein contaminants in the final LOS preparation, probably by the overlapping of lipooligosaccharides and DNase fractions.

In previous reports on LPS purification, DNase had to be eliminated before the chromatographic step, which complicates the process [11]. However, the use of mobile phases with a disaggregating effect could be useful to separate different subpopulations of LPS [23-25]. Two LPS subpopulations have been isolated from *E. coli* 055:B5 using size exclusion chromatography and the approach mentioned above [26]. On the other hand, LPS are found as micelles with a molecular weight of over 1000 kDa in an aqueous solution [22].

Considering the former criteria and taking into account the proposed objectives, it would be possible to select a mobile phase that could modulate the aggregation state of LPS. Since our main aim was to purify the LPS from nucleic acid, and because of its advantages, we decided to work with a mobile phase that would not affect the aggregation state of LPS in an aqueous solution. Any aqueous solvent without detergents or chelating agents could be used.

Although no comparisons with other aqueous phases were made, 0.2 M NaCl was selected because it is a very simple solvent and does not absorb at the selected wave length, while providing enough ionic force to avoid unspecific interactions between the sample and the stationary phase.

As expected 0.2 M NaCl did not affect the aggregation state of LPS and they eluted as a single fraction. This has the following advantages:

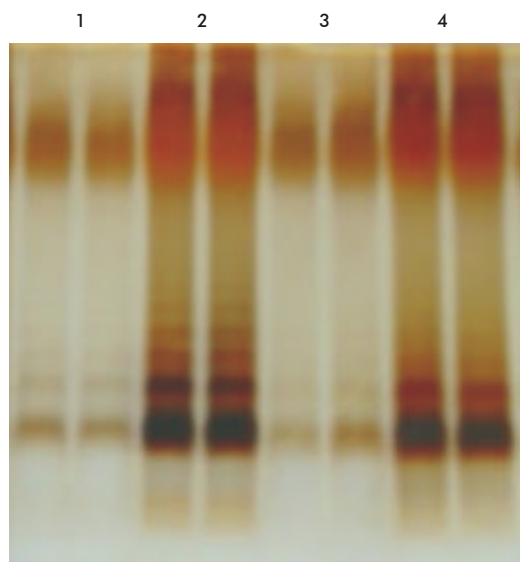


Figure 5. SDS-PAGE in 15% polyacrylamide gel. Lane 1: 5 mg/well of crude LPS; lane 2: 50 mg/well crude LPS; lane 3: 5 mg/well pure LPS; lane 4: 50 mg/well pure LPS. Each sample is duplicated.

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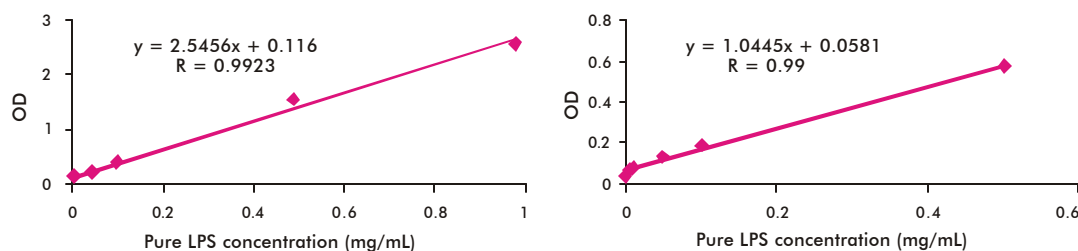


Figure 6. Linear correlation between LPS concentration and OD. Left panel: 206 nm. Right panel: 200 nm.

1) RNase and DNase treatments are not disadvantageous regarding the possible overlapping of enzymes and lipopolysaccharide fractions, because LPS elute in the void volume of the column ($K_{av} = 0$) and are clearly separated from nuclease fractions.

2) The elution of LPS in the void volume minimizes the dilution effect characteristic of size exclusion chromatography, and 3) it contributes to the separation of LPS from the oligonucleotides originated after the nuclease treatment, and reduces the possible presence of contaminant capsular polysaccharides.

Electrophoretic analysis (Figure 5) revealed a conserved pattern of bands for raw and pure LPS. This means that LPS are not chemically affected during the process and the number of subpopulations existing before purification was conserved.

We obtained a potency of 48.4 UE/ng for pure LPS, or a ten-fold increase compared to the potency of the control (raw LPS). This indicates that the purification protocol did not affect the biological potency of LPS. The increase in potency can be explained because of a purer product that contains a higher proportion of LPS per mass unit (UE/ng). The detection of LPS on the far UV spectrum was selected because most molecules absorb at these wavelengths. The absorption takes place because of electronic transitions in orbitals $n-p^*$ and $n-s^*$ derived mainly from functional carbonyl, carboxyl, amine and hydroxyl groups [27]. Seid and Sadoff [28] described the equivalence of KDO and absorption at 210 nm for the LPS fraction purified by liquid chromatography, which is the first report of LPS detection at the far UV spectrum.

Recently, a method for the characterization of *E. coli* 055:B5 LPS subpopulations by high-resolution capillary electrophoresis was described. [29]. This method employs the detection of absorbance at 200 nm of different LPS subpopulations. In the present work, a linear relationship between pure LPS concentration and optical density within a given concentration range (Figure 6) is demonstrated. Therefore, the Lambert-Beer law is thus fulfilled, and an analytical signal has been generated.

LPS are amphiphilic molecules, composed of a lipid region covalently bound to a polysaccharide moiety by an ester bond. The latter includes the specific O side chain and the central oligosaccharide, which contains 2-keto-3-deoxyoctulosonic acid (KDO). Lipid and polysaccharides are linked through KDO groups. The lipid component is composed of the disaccharide glucosamine, which is bound to fatty

acid chains (from four to six) by ester and amide bonds. [30].

Detection in the far UV spectrum has been widely used in the analysis and chromatographic purification of fatty acids [31]. Lipid A, which is present in all LPS, is the most highly conserved molecular moiety among strains of the same microorganism, and even among different species of gram negative bacteria [30], being therefore foreseeable that all LPS would absorb at these wave lengths. On the other hand it has been described that fatty acid lacking LPS [28], detoxified LPS (lipid A free) [29], and even bacterial polysaccharides [32], do absorb at the far UV spectrum. This suggests that both, the poly-saccharide moiety and the lipid A fatty acids, are capable of absorbing at wavelengths near 200nm.

The method described in this article was used to purify LPS extracts obtained through the Westphal method from *Escherichia coli* (EC01) wild strain and *Enterobacter agglomerans* (EA01) (Figure 7). LPS elute in the first fraction, well separated from nucleases and oligonucleotides. In both cases 98% pure LPS were obtained. When lyophilized pure LPS are dissolved in water and submitted to an UV spectrophotometer scanner (Figure 8), the maximum at 204 nm disappears (Figure 4a).

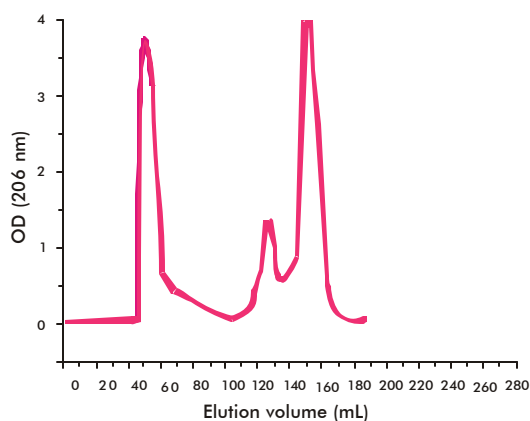


Figure 7. Chromatographic profile of raw *E. coli* (EC01) LPS. LPS extract (11 mL) at 21.2 mg/mL in 0.2 M NaCl, was incubated at 37 °C for two hours with RNase and DNase at a final concentration of 0.42 mg/mL (8.3 U/mL) and 42 µg/mL (117 U/mL), respectively. The hydrolysis was carried out at 65 °C for 12 min. Five mL of a 7 mg/mL LPS sample were injected. The chromatographic run was carried out in an XK 26/70 column at a flow rate of 9 cm h⁻¹.

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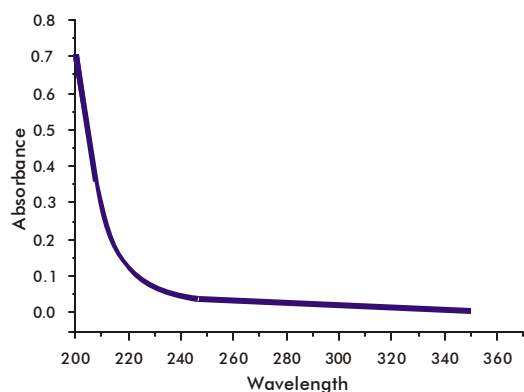


Figure 8. Absorption Spectrum of *E. coli* 055:B5 lyophilized pure LPS at 0.1 mg/mL dissolved in distilled water.

This effect was also observed for pure EA01 and EC01 LPS (Figure 9). This is due to the bathochromic effect (displacement of the absorption maximum toward higher wavelengths) caused by 0.2 M NaCl. This could represent an additional and unexpected advantage of the use of 0.2 M NaCl as the running buffer because it would increase sensitivity at the wavelength used. Further investigation is, however, required.

The specific or sensitive detection of LPS was not one of the goals of this study but the identification of relevant fractions during a purification process, where higher concentrations of LPS are generally handled. Other methods have been developed for the sensitive detection of LPS [33-34], among them is the Limulus Amebocyte Lysate (LAL), and the ELISA detection of proinflammatory cytokines in cell culture supernatants, isolated lymphocytes or whole blood.

The chromatographic profile of the process, monitored at either 206 nm or 260 nm is depicted in Figure 10. The ability of LPS and nucleic acid to absorb in the far UV spectrum enables the use of this spectrometric method to monitor the chromatographic run. The identification by UV absorption of the chromatographic fractions enables the immediate characterization of LPS and nucleic acids. Peptide bonds and aromatic amino acids also absorb at the far UV spectrum; therefore the use of these wavelengths also makes it possible to detect contaminant proteins that are usually present in LPS extracts purified by other methods [1-4]. Further studies would be warranted in order to evaluate if additional step using protease treatment, would be needed to completely eliminate contaminant proteins before the chromatographic procedure of extracts obtained using such methods.

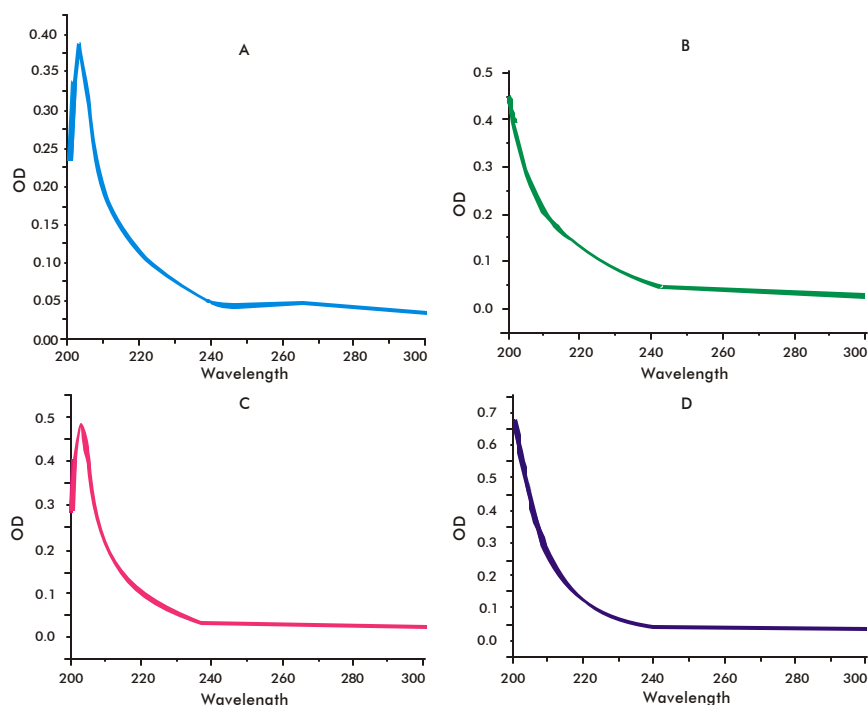


Figure 9. Spectrophotometric scanner from 200 to 300 nm of pure lyophilized LPS. A) EA01 LPS at 0.1 mg/mL in 0.2 M NaCl; B) EA01 LPS at 0.1 mg/mL in distilled water C) EC01 LPS at 0.1 mg/mL dissolved in 0.2 M NaCl; D) EC01 LPS at 0.1 mg/mL dissolved in distilled water.

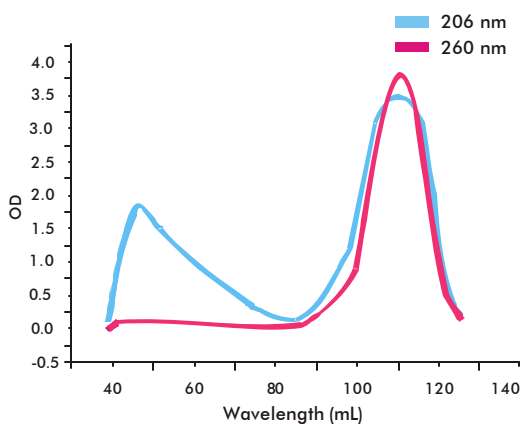


Figure 10 Chromatographic profile at 206 and 260 nm of LPS extract previously treated with both nucleases. Flow rate 9 cm h⁻¹, injection volume 2 mL at 10 mg/mL.

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