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Improved separation and characterization of lipopolysaccharide related compounds by reverse phase ion pairing-HPLC/electrospray ionization-quadrupole-mass spectrometry (RPIP-HPLC/ESI-Q-MS)

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ABSTRACT

A new approach for the separation and inline characterization of lipopolysaccharide (LPS) related compounds has been developed. The separation was based on the difference in the number of charged phosphate and ethanolamine groups, as non-stoichiometric substituents, on the polysaccharide backbone, and was achieved with reverse phase ion-paring chromatography (RPIP-HPLC). Tributylamine was used as an ion-pair reagent. In the conditions used in this study, tributylammonium then binds to the LPS related compounds through the negatively charged phosphate groups. This changes the hydrophobicity of the analytes at different positions and allows for separation based on both the number and position of the substituents on the analyte. The RPIP-HPLC was found to be effective for the separation of the *O*,*N*-deacylated derivative (deON) and polysaccharide portion (PS) from the LPS of *Escherichia coli* C strain. Post-column fluorescence derivatization (FLD), using sodium periodate and taurine, was used to detect the separated LPS related species. On the other hand, the separated species were also detected by direct infusion into the ESI-Q-MS using a volatile ammonium acetate buffer rather than the more traditional potassium phosphate buffer. The signal to noise ratio (S/N ratio) was low for the total ion chromatogram, however, high S/N ratios as well as good resolution were attained by selected ion monitoring (SIM) using *m*/*z* numbers corresponding to species with different numbers of non-stoichiometric substituents. Five species for deON and ten species for PS were clearly identified on the SIM chromatogram on the RPIP-HPLC/ESI-Q-MS. Accordingly, the present method allows for the effective separation and inline identification of the species corresponding to the diverse non-stoichiometric substitutions in LPS related compounds.

1. Introduction

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria. LPS stimulates innate immunity in a diversity of eukaryotic species [1]. It is composed of three distinct regions: lipid A, R-core, and *O*-antigenic polysaccharide [2]. The R-core region is further divided into the inner and outer R-core polysaccharides. The inner R-core and lipid A are substituted with phosphate and ethanolamine residues in non-stoichiometric ratios that affect the balance of static charges on the LPS molecule. The negatively charged phosphates strengthen the outer membrane by cross-linking the adjacent LPS molecules through a shared divalent cation, such as Ca^{2+}. Moreover, positively charged ethanolamines are present in strains resistant to the cationic antibiotic polymyxin [3,4].

Escherichia coli C is an Ra strain of *E. coli* with an R1 type core [5]. The R-core of the LPS is composed of two 3-deoxy-D-*manno*-octulosonic acid (KDO) and three L-*glycero*-D-*manno*-heptose (Hep) residues forming the inner R-core, and two galactose (Gal) and three glucose (Glc) residues forming the outer R-core (Fig. 1). However, the number and position of the non-stoichiometric substituents are variable and depend on the growing conditions. Previous studies have successfully demonstrated electrospray ionization-mass spectrometry (ESI-MS) can determine the diversity of such non-stoichiometric substitutions on LPS related compounds, such as *O*-deacylated (deO), *O*,*N*-deacylated (deON), and polysaccharide portion (PS), derived from the limited chemical degradation of the LPS [21]. The multiple species were detected for deON and PS derivatives by use of strongly basic anion exchange HPLC with post-column fluorescence derivatization (SAX-HPLC/FLD) and capillary zone electrophoresis with electrospray ionization ion-trap mass spectrometry (CZE/ESI-IT-MS) [6].

When SAX-HPLC has been used to analyze and fractionate deON and PS derivatives in the past [6], it has been difficult to inter-

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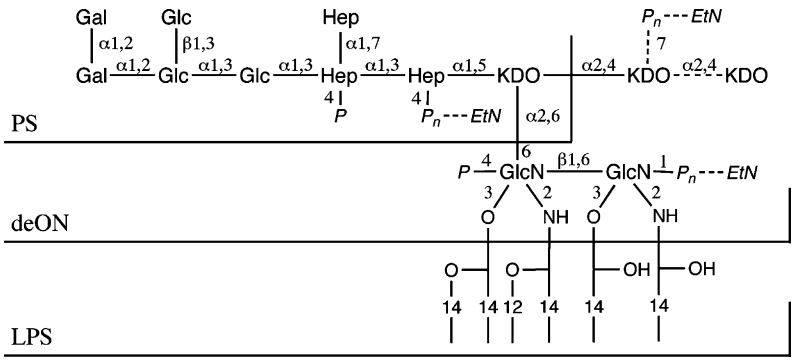

Fig. 1. Chemical structure of LPS of *E. coli* C strain and the positions of the limited chemical degradations to afford the *O*,*N*-deacylated LPS (deON) and polysaccharide portions of LPS (PS). Gal, D-galactose; Glc, D-glucose; Hep, L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-D-*manno*-octulosonic acid; GlcN, 2-amino-2-deoxy-D-glucose; *EtN*, ethanolamine; *P*, phosphate. Dotted lines indicate non-stoichiometric substitutions.

face with MS due to the high ionic strength of the mobile phase required to elute multiply charged oligosaccharides. Based on the salt formation with alkylamines combined with volatile mobile phases, reverse phase ion pairing-HPLC (RPIP-HPLC) has provided improved chromatographic resolution and MS compatibility [7]. This approach has been applied to the separation and characterization of oligosaccharides derived from hyaluronic acid [8] and of highly sulfated species from heparan sulfate [9] and heparin [10]. The RPIP-HPLC method should be applicable to the separation and characterization of the LPS related compounds. This report focuses on the separation of deON and PS derivatives of LPS based on the difference of the numbers of charged substituents by reverse phase ion pairing-chromatography (RPIP-HPLC) and the simultaneous characterization by the electrospray ionization-quadrupole-mass spectrometry (ESI-Q-MS) using selected ion monitoring.

2. Experimental

2.1. Materials

A Symmetry C18 column (3.5 μm, 2.1 mm ID × 50 mm) was obtained from Nihon Waters (Tokyo, Japan). Acetonitrile, tributylamine, ammonium acetate, acetic acid, taurine, sodium periodate, dipotassium hydrogen phosphate, sulfuric acid, and 28% ammonia solution were purchased from Wako Pure Chemical Industries (Osaka, Japan). Anhydrous hydrazine, phosphoric acid, and 48% potassium hydroxide solution were obtained from Sigma–Aldrich Japan (Tokyo, Japan). Sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, pyridine, petroleum ether, and potassium hydroxide (pellet), were purchased from Nacalai Tesque (Kyoto, Japan). Water was purified with a Milli-Q system from Nihon Millipore (Tokyo, Japan).

2.2. Samples

2.2.1. Lipopolysaccharide of E. coli C

The lipopolysaccharide (LPS) of *E. coli* C was extracted from cultured cells [11] by the phenol–chloroform–petroleum ether method [12].

2.2.2. Deacylated derivatives of LPS

The LPS of *E. coli* C was treated with anhydrous hydrazine (NH_2NH_2) at 37 °C for 30 min [13,14] to form *O*-deacylated LPS (deO). This was then treated with 4 M KOH at 125 °C for 18 h [15] to form *O*,*N*-deacylated LPS (deON). The polysaccharide part (PS) of LPS was obtained by hydrolyzing the LPS in 1% acetic acid at 100 °C for 2 h [13,16]. These degraded LPS derivatives were purified by gel filtration through Bio Gel P4 in a 1.5 cm ID × 110 cm column (Bio-Rad, Hercules, CA, USA) eluted with pyridine:acetic acid:water (10:5:1000, v/v/v) at pH 4.2. The saccharide containing fractions were detected by the phenol/H_2SO_4 method [17] or flow injection analysis using an inline HPLC fluorescence detector after derivatization using taurine and sodium periodate [18], and then combined and lyophilized.

2.3. Methods

2.3.1. Strongly basic anion-exchange HPLC/post-column fluorescence derivatization (SAX-HPLC/FLD)

All anion-exchange HPLC experiments were carried out on an LC-10AD_{VP} HPLC system from Shimadzu Co. Ltd. (Kyoto, Japan) consisting of three pumps, an auto sampler, a column oven, a degasser and a fluorescence detector. The chromatographic separations were carried out on a HiTrap Q HP column (1 mL) from GE Healthcare Bio-Sciences (Tokyo, Japan). The flow rate was 1.0 mL/min and the injection volume was 10 μL. Pump 1 was used to deliver 10 mM sodium phosphate buffer (pH 7.4) and pump 2 was used to deliver 10 mM sodium phosphate buffer (pH 7.4) containing 250 mM NaCl as a mobile phase. The mobile phase was composed of a mixture of pump 1 and 2. The gradient was delivered at 1 mL/min as follows: 0–5 min, 0% pump 2; 5–20 min, 0–100% pump 2; 20–25 min, 100% pump 2; 25–27 min, 100–0% pump 2; 27–35 min, 0% pump 2, giving a total loop time of 35 min. Pump 3 was used to deliver (0.3 mL/min) the post-column reagent, which was prepared from 12.5 g of taurine and 1.28 g of sodium periodate in 1000 mL purified water, and adjusted to pH 7.0 using sodium hydroxide pellets. A heating process for derivatization was performed at 130 °C in a hot air oven (CRB-6A) from Shimadzu Co. Ltd. (Kyoto, Japan). A cooling process was conducted at 35 °C in the same oven with a separate column. The reaction and cooling coil were 0.5 mm ID × 20 m and 0.5 mm ID × 10 m, respectively. The detection was performed at an excitation wavelength of 350 nm and an emission wavelength of 430 nm.

2.3.2. Reverse phase ion pairing-HPLC/post-column fluorescence derivatization (RPIP-HPLC/FLD)

An HPLC system from Agilent Technologies comprising of a Agilent 1100 HPLC system: binary pump 1 (lines 1 and 2) with a degasser for gradient analysis, auto sampler with sample cooling system, a column compartment for use as a column heater (left-hand side) and to cool the reaction mixture post-derivatization (right-hand side), a binary pump 2 (lines 1 and 2) with degasser for the post-column reagent and a fluorescence detector was used (Fig. 2, from a to b). *O*,*N*-Deacylated LPS (deON) of *E. coli* C and polysaccharide part of LPS (PS) were analyzed by ion pairing-HPLC separation using a 2.1 mm ID × 50 mm stainless steel Symmetry C18 column (3.5 μm, from Waters Ltd.). The flow rate was

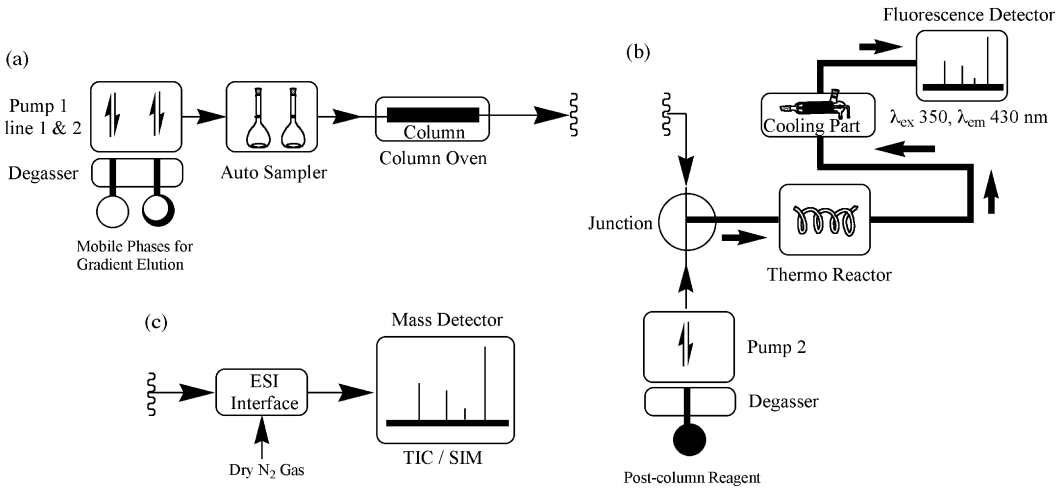

Fig. 2. RPIP-HPLC system with the post-column fluorescence derivatization (FLD) and electrospray ionization-quadrupole mass spectrometry (ESI-Q-MS).

0.25 mL/min and the injection volume was 5 μL. Binary pump 1 (line 1) was used to deliver tributylamine (15 mM) and phosphoric acid (15 mM) adjusted to pH 7.0 with 48% potassium hydroxide solution. Binary pump 1 (line 2) was used to deliver acetonitrile. The mobile phase was composed of the mixture of line 1 and line 2 of binary pump 1. The gradient was delivered at 0.25 mL/min as follows: 0 min, 5% pump 1 (line 2); 0–30 min, 5–30% pump 1 (line 2); 30–40 min, 30–60% pump 1 (line 2); 40–41 min, 60–5% pump 1 (line 2); 41–60 min, 5% pump 1 (line 2); giving a total loop time of 60 min for deON analysis and 0 min, 5% pump 1 (line 2); 0–30 min, 5–20% pump 1 (line 2); 30–40 min, 20–40% pump 1 (line 2); 40–41 min, 40–5% pump 1 (line 2); 41–60 min, 5% pump 1 (line 2); giving a total loop time of 60 min for PS analysis. Binary pump 2 (line 1) was used to deliver (0.25 mL/min) the post-column reagent, which was prepared from 12.5 g of taurine and 1.28 g of sodium periodate in 1000 mL purified water. HPLC elution fraction was mixed with the post-column reagent at a T-junction and the mixture was introduced into the Shiseido thermo reactor (model 3019). A heating process for derivatization was performed in the thermo reactor. The reaction coil was 0.25 mm ID × 10 m. A cooling process was conducted in the same column compartment with the same column. The cooling volume was 5 μL. The column compartment was maintained at 30 °C for the separation on the left-hand side and at 20 °C on the right-hand side for the cooling process. The detection was performed at an excitation wavelength of 340 nm and an emission wavelength of 430 nm. The software version was Chem Station Rev. A08.03 (Agilent Technologies, Inc., USA).

2.3.3. Reverse phase ion pairing-HPLC/electrospray ionization-quadrupole-mass spectrometry (RPIP-HPLC/ESI-Q-MS)

RPIP-HPLC/ESI-Q-MS analysis was performed in the negative ion mode using a Waters 2695 HPLC system, composed of a quaternary pump, an auto liquid sampler, and a thermostatted column compartment, coupled with a Micromass ZQ2000 system (Waters, USA) (Fig. 2, from a to c). DeON and PS derivatives were analyzed by ion pairing-HPLC separation using a 2.1 mm ID × 50 mm stainless steel column Symmetry C18 (3.5 μm, from Waters Ltd.). The flow rate was 0.25 mL/min and the injection volume was 5 μL. The quaternary pump line 1 was used to deliver triethylamine (15 mM) and acetic acid (15 mM) adjusted to pH 7.0 with 28% ammonia solution. The quaternary pump line 2 was used to deliver acetonitrile. The mobile phase was composed of the quaternary pump line 1 and 2. The gradient was delivered at 0.25 mL/min as follows: 0 min, 5% line 2; 0–30 min, 5–30% line 2; 30–40 min, 30–60% line 2; 40–41 min, 60–5% line 2; 41–60 min, 5% line 2; giving a total loop time of 60 min for deON analysis and 0 min, 5% line 2; 0–30 min, 5–20% line 2; 30–40 min, 20–40% line 2; 40–41 min, 40–5% line 2; 41–60 min, 5% line 2, giving a total loop time of 60 min for PS analysis.

Mass spectra were acquired using a Micromass ZQ2000 system (Waters, USA). Capillary entrance voltage was set to 3.0 kV, the dry gas temperature was 350 °C, and the cone voltage was varied from −10 to −60 V. The mass range was set to 500–2000 *m*/*z* for scanning mode and in accordance with Table 1 for selected ion monitoring mode (SIM mode). A software version was MassLynx version 4.1 (Waters, USA).

2.3.4. Capillary zone electrophoresis/electrospray ionization-ion-trap-mass spectrometry (CZE/ESI-IT-MS)

Agilent standard interfaces including gas-junction, liquid-junction, and coaxial liquid sheath-flow were used to couple the CZE to the ESI-IT-MS. An Agilent 3D-CE system was coupled to an Agilent Ion-Trap mass spectrometer via a CZE-ESI interface. The sheath solution was delivered to the liquid-junction of the CZE-ESI interface using a binary pump with a splitter (1:100) and peek tube (0.25 mm ID). Ammonium acetate (10 mM, pH 9.0) was used as the running electrolyte and the electrophoresis was carried out at 30 kV for both the deON and PS derivatives. In the CZE/ESI-IT-MS system, ca 10 nL of sample was typically injected at 50 mbar for a duration of 10 s. The composition and flow rate of the sheath solution was aqueous 50% methanol and 5 μL/min.

Mass spectra were acquired using an Agilent Ion-Trap mass spectrometer (Agilent Technologies, Inc., USA), with a scanning resolution of 13000 *m*/*z*/s in a standard scanning range (200–2200 *m*/*z*). The electrospray interface was used in negative ionization mode with the capillary voltage at 3.5 kV and a heat source of 300 °C. Nitrogen was used as the drying (10 L/min) and nebulizing gas (2.5–10 psi). Software versions were MSD trap control version 5.1 and Data Analysis version 2.2 (Agilent Technologies, Inc., USA).

3. Results and discussion

3.1. Development of the separation mode from strongly basic anion exchange (SAX) to reverse phase ion pairing (RPIP) chromatography

Attempts to separate and characterize the LPS related compounds of the *E. coli* C strain, such as *O*,*N*-deacylated LPS (deON) and

Table 1
Selected and observed negative ions on RPIP-HPLC/ESI-Q-MS for the deON and PS derivatives.

Sample	Species	Proposed composition	Molecular mass (Da)	Selected ion (m/z)		Retention time (min)	Rel int (%)
			Calculated	$[M-3H]^{3-}$	$[M-2H]^{2-}$		
deON	deON 3P	Hex_5 Hep_3 KDO_2 $HexN_2$ P_3	2406.6	801.2		23.2	6
	deON 4P	Hex_5 Hep_3 KDO_2 $HexN_2$ P_4	2486.7	827.9		26.0	100
	deON 5P	Hex_5 Hep_3 KDO_2 $HexN_2$ P_5	2566.5	854.5		27.7	58
	deON + EtN 3P	Hex_5 Hep_3 KDO_2 $HexN_2$ P_3 EtN	2449.8	815.6		–	N.D.
	deON + EtN 4P	Hex_5 Hep_3 KDO_2 $HexN_2$ P_4 EtN	2529.6	842.2		–	N.D.
	deON + EtN 5P	Hex_5 Hep_3 KDO_2 $HexN_2$ P_5 EtN	2609.7	868.9		26.3	24
	deON-KDO 3P	Hex_5 Hep_3 KDO $HexN_2$ P_3	2186.7	727.9		–	N.D.
	deON-KDO 4P	Hex_5 Hep_3 KDO $HexN_2$ P_4	2266.5	754.5		24.9	8
	deON-KDO 5P	Hex_5 Hep_3 KDO $HexN_2$ P_5	2346.6	781.2		–	N.D.
PS	PS 1P	Hex_5 Hep_3 KDO P	1704.4		851.2	12.5	5
					851.2	14.9	5
	PS 2P	Hex_5 Hep_3 KDO P_2	1784.4		891.2	27.0	100
	PS 3P	Hex_5 Hep_3 KDO P_3	1864.4		931.2	30.5	39
					931.2	32.6	8
	PS 4P	Hex_5 Hep_3 KDO P_4	1944.4		971.2	–	N.D.
	PS + EtN 2P	Hex_5 Hep_3 KDO P_2 EtN	1827.4		912.7	–	N.D.
	PS + EtN 3P	Hex_5 Hep_3 KDO P_3 EtN	1907.4		952.7	23.3	44
	PS + EtN 4P	Hex_5 Hep_3 KDO P_4 EtN	1987.4		992.7	–	N.D.
	PS 2P–H_2O	Hex_5 Hep_3 KDO P_2-H_2O	1766.4		882.2	27.4	86
						27.8	26
	PS 3P–H_2O	Hex_5 Hep_3 KDO P_3-H_2O	1846.4		922.2	30.5	28
						30.9	13
	PS 4P–H_2O	Hex_5 Hep_3 KDO P_4-H_2O	1926.4		962.2	–	N.D.
	PS + EtN 2P–H_2O	Hex_5 Hep_3 KDO P_2 EtN-H_2O	1809.4		903.7	–	N.D.
	PS + EtN 3P–H_2O	Hex_5 Hep_3 KDO P_3 EtN-H_2O	1889.4		943.7	23.3	44
	PS + EtN 4P–H_2O	Hex_5 Hep_3 KDO P_4 EtN-H_2O	1969.4		983.7	–	N.D.

Average mass units were used for calculation of molecular mass values based on proposed compositions as follows: Hex, 162.14; Hep, 192.17; KDO, 220.18; HexN, 161.16; P, 79.98; EtN, 43.04. N.D.: not detected.

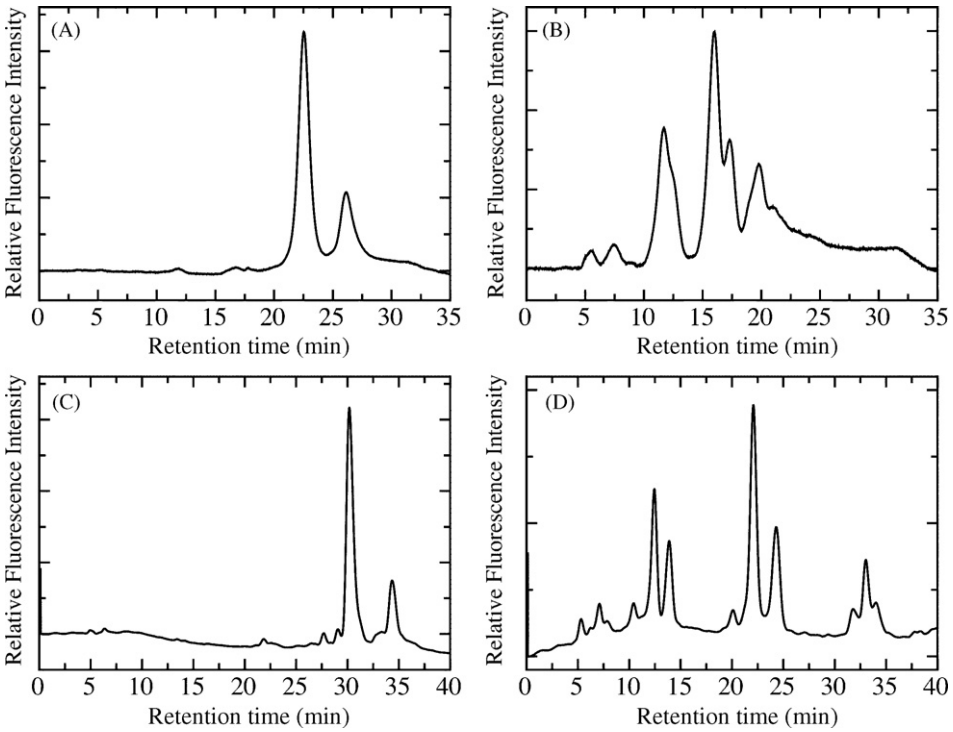

Fig. 3. Separation of the *O*,*N*-deacylated LPS (A and C) and polysaccharide portion of LPS (B and D) by strongly basic anion-exchange chromatography with post-column fluorometric derivatization (SAX-FLD) (upper) and reverse phase ion pairing-chromatography with post-column fluorometric derivatization (RPIP-FLD) (lower). The HPLC conditions for SAX were: column, HiTrap Q HP (1.0 mL); flow rate, 1.0 mL/min; eluent, 10 mM sodium phosphate (pH 7.0) with programmed liner gradient 5–25 min with sodium chloride 0–250 mM; post-column reagent, 100 mM taurine and 6 mM sodium phosphate (pH 7.0); post-column reagent flow rate, 0.3 mL/min, fluorescence detection, λ_{ex} 350 nm and λ_{em} 430 nm. The HPLC conditions for RPIP were: column, Waters Symmetry C18 (particle size 3.5 μm, 2.1 mm ID × 50 mm); flow rate, 0.25 mL/min; eluent, 15 mM acetic acid containing 15 mM tributylamine adjusted at pH 7.0 with 45% KOH solution; programmed acetonitrile gradient, 5–30% at 0–30 min and 30–60% at 30–40 min for *O*,*N*-deacylated LPS (C) and 5–20% at 0–30 min and 20–40% at 30–40 min for polysaccharide portion of LPS (D); post-column reagent, 100 mM taurine and 6 mM sodium phosphate (pH 7.0); post-column reagent flow rate, 0.25 mL/min, fluorescence detection, λ_{ex} 350 nm and λ_{em} 430 nm.

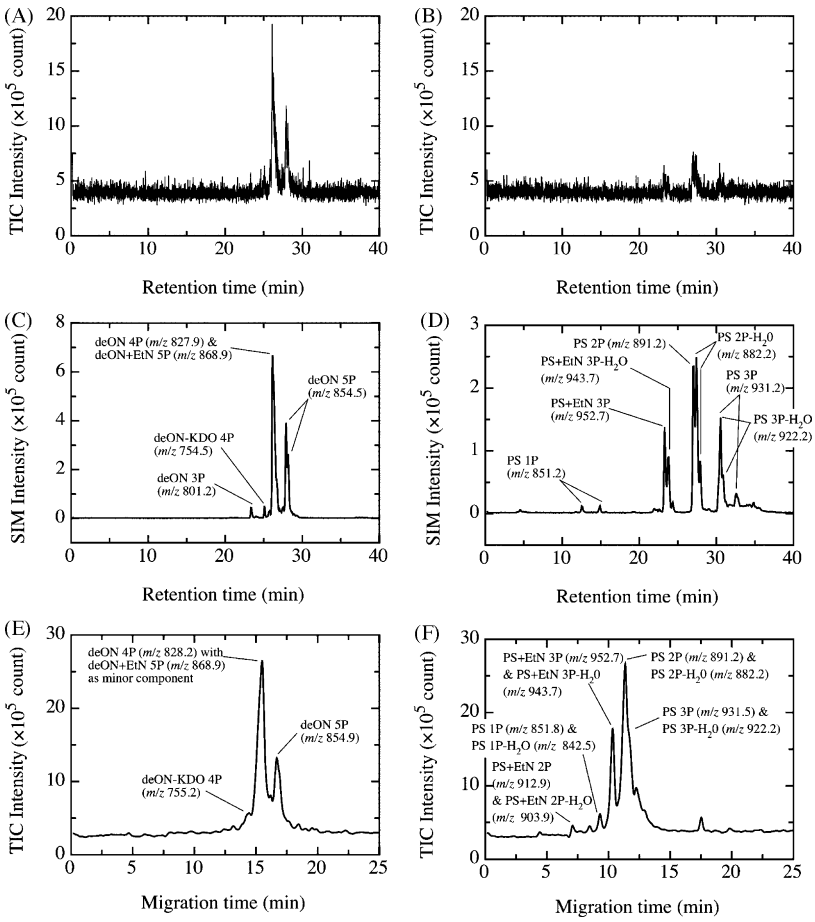

Fig. 4. Separation of *O*,*N*-deacylated LPS (A, C and E) and polysaccharide portion of LPS (B, D and F) analyzed by the reverse phase ion pairing-chromatography with electrospray ionization-quadrupole mass spectrometry (RPIP/ESI-Q-MS) with total ion monitoring (A and B) and with selected ion monitoring (C and D) and by the capillary zone electrophoresis with electrospray ionization ion-trap mass spectrometry (CZE/ESI-IT-MS) with total ion electropherogram (E and F). The RPIP-HPLC conditions were: column, Waters Symmetry C18 (3.5 μm, 2.1 mm ID × 50 mm); flow rate, 0.25 mL/min; eluent, 15 mM acetic acid containing 15 mM tributylamine adjusted at pH 7.0 with 28% ammonia solution; programmed acetonitrile gradient, 5–30% at 0–30 min and 0–60% at 30–40 min for the *O*,*N*-deacylated LPS (A and C) and 5–20% at 0–30 min and 20–40% at 30–40 min for the polysaccharide portion of LPS (B and D). The ESI-Q-MS conditions were: scanning range, 500–2000 *m*/*z* or selected ion monitoring mode. The CZE conditions were: capillary, fused silica capillary (50 μm ID × 100 cm); running electrolyte, ammonium acetate (10 mM, pH 9.0); electrophoresis, 30 kV. The ESI-IT-MS scanning range was 200–2200 *m*/*z* [6].

polysaccharide portion of LPS (PS) (Fig. 1) showed the compounds were not retained on standard reverse phase columns. A strongly basic anion-exchange column with quaternary ammonium group (Hi TrapQ HP) retained the deON and PS derivatives and they could be eluted later with an increasing sodium chloride gradient. They are separated based on the number of charged phosphate and protonated ethanolamine substituents (Fig. 3A and B) [6]. Since the LPS related compounds have no chromophore, the compounds were detected by post-column derivatization using taurine and sodium periodate [18,19]. Unfortunately, further control of the separation energy was difficult by SAX-HPLC, as it involved a simple charge on-off mode. The key to the separation of LPS related compounds was thought to be in the number of the phosphate residues. Accordingly, a hydrophobic ion-pair reagent was employed in order to introduce hydrophobic properties to the compounds.

Reverse phase chromatography using 15 mM tributylamine as an ion-pair reagent and potassium phosphate buffer as mobile phase (RPIP-HPLC) accomplished retention of the deON and PS derivatives onto the column. Gradient elution of the species contained on the column was achieved (Fig. 3C and D) using acetonitrile as the organic modifier. The separated species were also detected by the above-mentioned post-column derivatization system (Fig. 2, from a to b). In this system, the separation capacity and sensitivity were much improved by the use of semi-micro HPLC equipment, which reduced the path of flow from 0.5 to 0.25 mm ID, compared with SAX-HPLC (Fig. 3A and 3B). Moreover, enhancing the efficiency of cooling after the post-column reaction increased the sensitivity at the fluorescence detector.

The deON derivative, which was separated into two peaks on the SAX-HPLC (Fig. 3A), was further divided into two major and two minor peaks on the RPIP-HPLC (Fig. 3C). The PS derivative was separated into multiple peaks, as shown in Fig. 3B, on the SAX-HPLC. Fig. 3D shows this sample could be separated more efficiently on RPIP-HPLC. By introducing an ion-pair reagent, the number of charged substituents on the different species was converted into a difference in the hydrophobicity. Furthermore, different positions of the charged substituents caused differences in the distribution of the hydrophobic areas.

3.2. Separation of deON and PS derivatives by reverse phase ion pairing-chromatography and subsequent detection by electrospray ionization-quadrupole-mass spectrometry (RPIP-HPLC/ESI-Q-MS)

The deON and PS derivatives were indeed separated and detected by the RPIP-HPLC/FLD system (Fig. 2, from a to b). How-

ever direct injection of these fractions into a mass spectrometer as they eluted was not possible due to the high concentration of salt in the elution buffer. Thanawiroon et al. [20] reported on the RPIP-HPLC analysis of heparin oligosaccharides by using an MS-friendly mobile phase including the volatile ion-pair reagent tributylamine, the volatile salt ammonium acetate, and acetonitrile as the organic modifier. Consequently, the separation buffer was changed to one composed of 15 mM tributylamine and 15 mM acetic acid adjusted to pH 7.0 with 28% aqueous ammonia for the RPIP-HPLC/ESI-Q-MS system (Fig. 2, from a to c).

The total ion chromatograms (TICs) of the deON and PS derivatives in negative ion mode (m/z 500–2000) are illustrated in Fig. 4A and B, respectively. The derivatives were separated within 40 min using a linear acetonitrile gradient. The use of the volatile ammonium tributylammonium acetate buffer instead of the potassium tributylammonium phosphate system, caused some debasement of the separation. The S/N ratio was rather low and only the main species of deON and PS derivatives could be detected in the chromatograms. Accordingly, selected ion monitoring (SIM) was employed to improve the detectability by using the calculated m/z values of different species with different numbers of charged substituents on the polysaccharide backbones of the deON and PS derivatives (Table 1). The selected ion chromatograms of RPIP-HPLC/ESI-Q-MS of the deON and PS derivatives showed sharp and intense, well resolved peaks (Fig. 4C and D).

In Fig. 3C, deON 3P and deON-KDO 4P were also clearly detected in addition to two main species of deON 4P and deON 5P. In the case of deON 5P, two peaks were detected on the SIM. In the PS sample (Fig. 3D), PS 1P-3P and PS + EtN 3P were also detected. In the cases of PS 1P and PS 3P, two peaks were detected in the SIM. Some positional isomers of the charged substituents within the deON and PS derivatives could have been separated in the RPIP-HPLC/ESI-Q-MS. These results indicate that reverse phase ion pairing-chromatography is a highly effective method for the analysis of the diversity of non-stoichiometric substituents on LPS related compounds. In addition, peaks corresponding to the dehydration products were also observed. These dehydration products were detected in our previous study of the ESI-Q-MS analysis of the purified PS derivative [21]. In that study it was not clear whether these were generated during the acid hydrolysis of LPS or during the MS measurement. Consequently, the present experiment confirms that they were generated during hydrolysis. The cone voltage in the ESI-Q-MS was varied from −10 to −60 V in order to obtain further information on the positions of non-stoichiometric substitutions, however, the fragmentation of the separated species was not promoted.

In Fig. 4E and F, the results of the analysis of the same compounds by CZE/ESI-IT-MS are shown as comparison. The SIM chromatograms from the RPIP-HPLC/ESI-Q-MS method were able to improve the sharpness of separation, and to identify more species compared with the total ion electropherograms (TIEs) by the CZE/ESI-IT-MS method. In the CZE analysis, the total charge and the effective hydration radius greatly influenced the separation. The effective hydration radius the LPS related compounds are not particularly different because they all have the same polysaccharide backbone. Thus, the driving force for the separation is solely from the differences in the total charge. However, CZE/ESI-IT-MS can achieve relatively high sensitivity from very small sample sizes by optimizing of composition of the sheath solution [6]. A high performance anion-exchange chromatography (HPAEC) with pulsed amperometric detector (PAD) is also successfully applied to the analysis of the carbohydrates [22,23]. Due to high salinity and low volatility, alkaline hydroxide and acetate eluents were incompatible to mass spectrometry. Thus, an elaborate electrochemical desalting is under studying to interface the HPAEC with MS [24–26]. The present RPIP-HPLC/ESI-Q-MS also had to admit a lower sensitivity than CZE/ESI-IT-MS due to the addition of an ion-pair reagent. On the other hand, RPIP-HPLC is more capable at separating the diverse species of LPS related compounds as it converts the differences in the number and position of the charged residues into differences in the size and distributions of the hydrophobicity. Moreover, the present method has an advantage that highly reproducible results are feasible because of the easy to operate HPLC system. Characterization of the separated species through MS was indeed possible, however, the precise reproducibility and accuracy for quantitative analysis was not so much effective at the present stage of investigation. Thus, the combination and selection of the above methods can provide better information on the diversity of non-stoichiometric substituents on LPS. The MS/MS measurements were challenged for the separated species by CZE and RPIP-HPLC using ESI-Q/Q-MS/MS and ESI-IT-MS, however, MS sensitivity was drastically reduced compared to the measurement using a simple ESI-Q-MS. This would be a subsequent step of the investigation. The non-stoichiometric charged substitutions on the lipopolysaccharide backbone affect the biological potential of LPS. The present analytical method is valuable for the researchers on the area that the biological activity of LPS would be changed by such non-stoichiometric substitutions to overview their diversity and to evaluate the interaction for example in a frontal analysis with protein and antibody.

4. Conclusion

The separation of deON and PS derivatives was improved by RPIP-HPLC coupled with fluorometric detection using post-column derivatization (FLD). The separated species in the derivatives were directly analyzed by coupling with ESI-Q-MS. Selected ion monitoring (SIM) using the specific m/z values which correspond to the species having different numbers of phosphate and ethanolamine residues determined the composition of the diverse species among the deON and PS derivatives. The present analytical method is one of the most successful applications, and offers high resolution as well as precise characterization of the diverse non-stoichiometric substitutions of LPS related compounds.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.12.028.

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