Ph. D. Thesis

Development of Analytical Method and its Application for Lipopolysaccharide

（リポ多糖の分析手法の開発と応用）

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Contents

Abbreviations

LPS	lipopolysaccharide
Glc	glucose
Gal	galactose
GlcN	glucosamine
GlcNAc	*N*-acetyl glucosamine
Hep	L-*glycero*-D-*manno*-heptose
KDO	3-deoxy-D-*manno*-octulosonic acid
P	phosphate
EtN	ethanolamine
deO	*O*-deacylated LPS
deON	*O,N*-deacylated LPS
PS	polysaccharide portion of LPS
HF	hydrofluoric acid
ESI-Q-MS	electrospray ionization quadrupole mass spectrometer
HPLC	high performance liquid chromatography
SAX	strongly basic anion-exchange chromatography
CZE/ESI-IT-MS	capillary zone electrophoresis/ESI-ion trap-mass spectrometer
RPIP-HPLC	reverse phase ion-paring chromatography
FLD	fluorescence derivatization
SIM	selected ion monitoring
SEC	size exclusion chromatography
EOF	electroosmotic flow
IT-MS	ion trap-mass spectrometry
TIE	total ion electropherogram
EIE	extracted ion electropherogram

General Introduction

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria such as *Escherichia coli, Salmonella, Shigella, Pseudomonas, Neisseria, Haemophilus*, and other leading pathogens [1]. It is composed of three distinct domains: lipid A, R-core, and *O*-antigenic polysaccharide (Fig. 1A) [2]. Lipid A is a glucosamine-based glycolipid that serves the hydrophobic, membrane-anchoring region of LPS, consisting of a phosphorylated *N*-acetyl glucosamine dimer with six or seven saturated fatty acids attached [3]. The structure of the lipid A is highly conserved among gram-negative bacteria. On the other hand, the R-core, which is composed of the inner core and the outer core, and various kinds of non-repeating oligosaccharide units. The outer R-core has mainly hexose such as glucose (Glc), galactose (Gal), glucosamine (GlcN), and *N*-acetyl glucosamine (GlcNAc). The inner R-core usually has two unusual sugars such as D or L-*glycero*-D-*manno*-heptose (Hep) and 3-deoxy-D-*manno*-octulosonic acid (KDO) [4]. The *O*-antigenic polysaccharide also referred to as the *O*-antigen is attached to the outer core polysaccharide, consisting of repeating oligosaccharide subunits made up of three to five sugars. The individual chains vary in length up to 40 repeat units [5], maintaining the hydrophilic domain of the LPS molecule. The LPS can be classified into a smooth or rough type LPS depending on whether the *O*-antigen is attached to its R-core. The strain of *E. coli* C has a rough type LPS (Fig. 1B). The structure of the R-core represents the *E. coli* R1 type core containing Gal, Glc Hep, and KDO [2-5]. The non-stoichiometric substituents are attached on the inner R-core and lipid A include phosphate (*P*), ethanolamine (*EtN*), and an additional third KDO group, which affect the balance of static charges of the LPS molecule. The negatively charged phosphates contribute to strengthening the outer membrane by cross-linking adjacent LPS molecules while sharing a divalent cation such

as Ca^{2+}. The positively charged protonated amines help to provide resistance to cationic antibiotics such as polymyxin [1,6]. These and other non-stoichiometric substitutions can result in changes to the pathogenicity of many gram-negative bacteria [7,8].

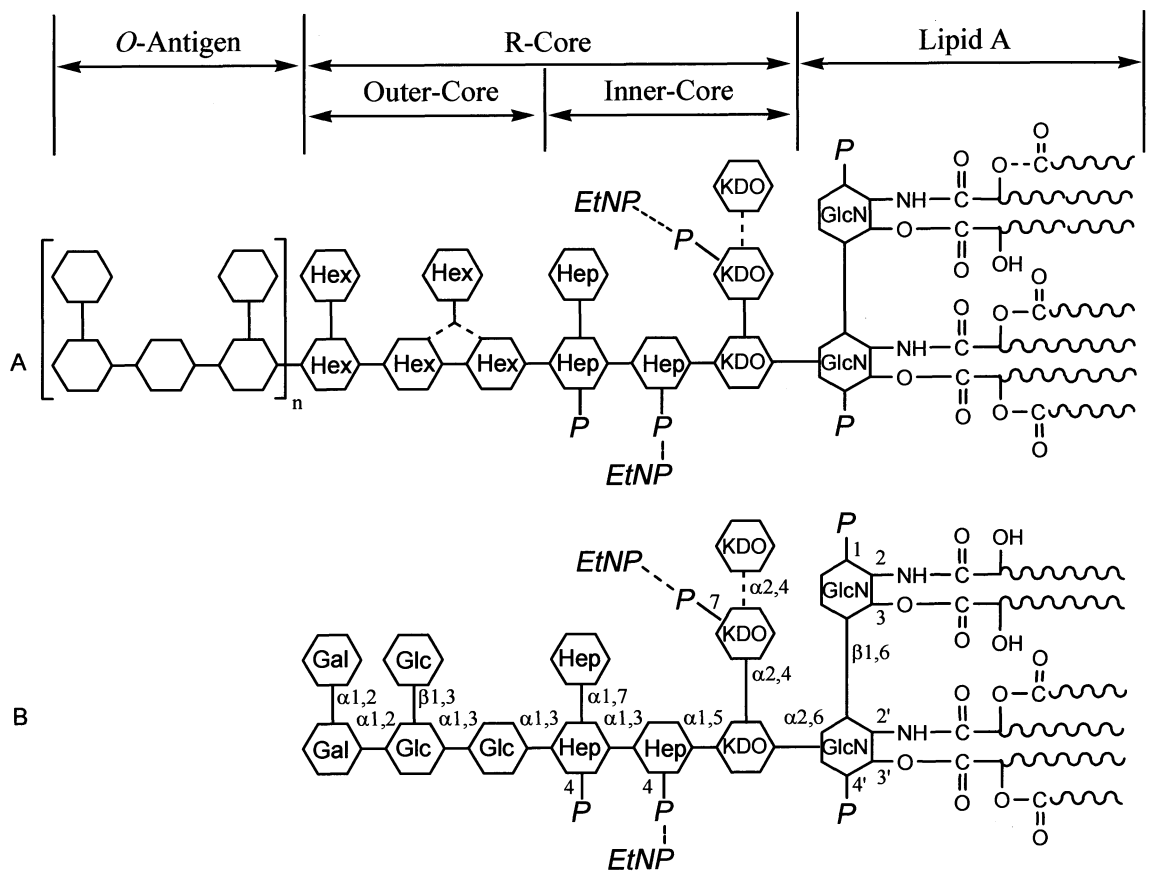


Fig. 1. Schematic structure of the LPS of enterobacteria (A) and *E. coli* C (B).
Abbreviations are: Hex, hexose; Hep, D or L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-D-*manno*-2-octulosonic acid; GlcN, 2-amino-2-deoxy-D-glucose; Gal, D-galactose; Glc, D-glucose; *P*, phosphate; *EtN*, 2-aminoethanol.

LPS is known as endotoxin to have potent biological effects in humans. Serious endotoxin intoxication can cause sepsis and septic shock, leading to severe hypertension, cardiovascular collapse, multiple organ failure and death [9]. In the biotechnology industry Gram-negative bacteria are widely used to produce recombinant DNA products such as peptides and proteins. Bacterial endotoxin has been recognized by the industry as a major cause of the pyrogenic reactions that can be encountered during the administration of biotherapeutics [10]. The removal of these physiologically active agents from final bioproducts has always been a challenge, especially in situations where endotoxin binds to the bioproducts such as polysaccharides and proteins.

LPS is also known to be receptor for bacteriophage. Bacteriophage ϕX174 is a small icosahedral virus that consists of a single-stranded circular DNA and four capsid proteins, designated F, G, H and J [11]. The phage has a spike protrusion composed of five major spike G proteins and one minor spike H protein [12] at each of its icosahedral vertices, and adsorbs onto host enterobacteria, such as *E. coli* C, through these spikes [13]. Thus, the spike proteins have been considered to recognize LPS, a major component of the outer membrane of enterobacteria, as a receptor for infection. The interactions of the G [14] and H [15] proteins with LPS were analyzed quantitatively by their fluorescence spectra and circular dichroism (CD) in order to determine their binding parameters.

The author has also reported that the non-stoichiometric substitution on LPS also affected the ability of the receptor for bacteriophage ϕX174. The affinity for the phage proteins was decreased when the LPS was first treated with hydrofluoric acid (HF) for removal of the phosphate residues and/or sodium borohydride ($NaBH_4$) combined with water soluble carbodiimide (WSC) for reduction of the carboxylic acid moiety on the KDO residues [8]. Conventional colorimetric methods using molybdenum blue [8] or thiobarbituric acid [8] indicated that there were 5.1 residues of

phosphate and 2.2 residues of KDO were present in the intact LPS preparation, and that the lower amounts of the above residues in the treated LPS preparations. Thus, additional information is needed in order to identify the contributions of the LPS substituents to the recognition mechanism of bacteriophages. In order to elucidate the relationship between the phage proteins and the LPS substituents, the first study is structure analysis; investigation of the diversity of non-stoichiometric substitutions of LPS from *E. coli* C is very important. The second study is to develop the analytical method for LPS related compounds.

Research Strategy

The methodology of electrospray ionization quadrupole mass spectrometer (ESI-Q-MS) analysis is suitable for component analysis and microanalysis of LPS related compounds which are difficult to get the much of purified sample. Thus, we have decided that method development of sensitive analysis is valuable for the research of LPS related compounds. Throughout of this thesis, we chose the following methodology. The LPS related compounds were purified by gel filtration through a Bio Gel P4 column and then they were lyophilized. If the obtained LPS related compounds are pure in terms of contamination of inorganic salts such as sodium phosphate and sodium chloride, ESI-Q-MS analysis can be used with high sensitivity. The resulting diversity of non-stoichiometric substituents on the inner R-core of *E. coli* C LPS were revealed. The methods for chromatographies such as ion-exchange column, reversed phase HPLC, and capillary zone electrophoresis, were conducted to develop the method of separation of the LPS related compounds. Combination of the results of the LPS related structures allow us to construct novel systems for the chemical structure and the biosynthesis of LPS from enterobacteria.

Survey of this thesis

Research works have been carried according to the following projects.

In chapter 1, the diversity of non-stoichiometric substitutions on chemically degraded derivatives of LPS from *E. coli* C was investigated. The LPS of *E. coli* C (NBRC 13898) was extracted from the cultured cells [16] using a phenol–chloroform–petroleum ether method. [17] The extracted LPS was submitted to limited chemical degradations [14] according to the reported method with anhydrous hydrazine (NH_2NH_2) at 37°C for 30 min to form *O*-deacylated LPS (deO) and then further treated with 4 M KOH at 125°C for 18 h to form *O,N*-deacylated LPS (deON). The polysaccharide part of LPS (PS) was obtained by hydrolyzing the LPS in 1 % acetic acid at 100°C for 2 h. The degraded LPS derivatives were purified by gel filtration through a Bio Gel P4 column (1.5 cm I.D. × 110 cm) (Bio-Rad, Hercules, CA, USA) eluted with pyridine/acetic acid/H_2O (10:5:1000 v/v/v at pH 4.2). The saccharide-containing fractions were detected using a phenol-H_2SO_4 method [17] or flow injection analysis in line with a HPLC fluorescence detector after derivatization using taurine and sodium periodate [18] and then they were combined and lyophilized. The diversity of non-stoichiometric substitutions on chemically degraded derivatives of LPS from *E. coli* C was analyzed by electrospray ionization-single quadrupole-mass spectrometry (ESI-Q-MS).

In chapter 2, the *O,N*-deacylated derivative (deON) and polysaccharide part (PS) from the lipopolysaccharide (LPS) of *E. coli* C strain were separated by strongly basic anion-exchange chromatography (SAX) based on the differences in the number of charged phosphate and ethanolamine substituents. They were also successfully separated and characterized by capillary zone electrophoresis and subsequent ESI-ion

trap-MS (CZE/ESI-IT-MS) by means of selection of a sheath solution for separation on CE, and optimization of ionization conditions on IT-MS. And the good analytical results were obtained on the deON and PS derivatives. Moreover, from the *O*-deacylated derivative (deO) which was presented as a broad peak in CZE/ESI-IT-MS, more than twelve species could be discriminated by an extracted ion electropherogram (EIE) monitoring the species which have different numbers of phosphate and ethanolamine substituents on polysaccharide backbone.

In chapter 3, 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, tributyl ammonium 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.

In chapter 4, HPLC and post column fluorescence derivatization was applied for LPS analysis. HPLC/MS analyses of intact LPS are very difficult because of their physical property because of formation of huge aggregates in aqueous solution. However negatively-charged phosphate residues can be neutralized with ion-pair formation by the addition of positively charged trialkyl ammonium cation. The resulted paried molecule can be handled as large aggregate. And the lump was separable in the mode of size exclusion chromatography (SEC). Additionally, post column fluorescence derivatization using sodium periodate and taurine were effective for the detection of LPS which have no chromophore. A sensitive size-exclusion chromatography (SEC) method has been developed with post column fluorescence derivatization using sodium periodate and taurine for the visualization of lipopolysaccharide (LPS) samples from a series of *Salmonella* mutants and several *Escherichia coli* serotypes, such as O111:B4, C, J-5, and F583. This method can detect as little as 0.1-3 μg of LPS depending on the number of component sugar having *vicinal*-diol structure. These LPSs can be separated from small molecules, such as glucose, maltose, and α-cyclodextrin (α-CD). This was utilized to monitor quantitatively the total LPS content.

Chapter 1

Diversity of non-stoichiometric substitutions on the lipopolysaccharide of *E. coli* C demonstrated by electrospray ionization single quadrupole mass spectrometry

Abstract

The lipopolysaccharide (LPS) of enterobacteria frequently contains various numbers of charged non-stoichiometric substituents such as phosphate (*P*) and ethanolamine (*EtN*) groups and a third residue of 3-deoxy-D-*manno*-2-octulosonic acid (KDO) on the R-core polysaccharide backbone. These substituents can modify the biological activities of LPS including varying the stability of the outer membrane, tolerance to cationic antibiotics, pathogenicity, and sensitivity to enterobacteria bacteriophages. These diverse substituents can be clearly detected in degraded samples of LPS from *E. coli* C using ESI-Q-MS from a 0.1 mg/mL solution in a 50:50 mixture of methanol and 10 mM ammonium acetate (pH 6.8). The *O*-deacylated derivative showed multiple peaks of $[M-3H]^{3-}$ ions which corresponded to species having up to 8 phosphates, 2-ethanolamines, and an additional KDO on the backbone of Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$. The major components of the *O,N*-deacylated derivative were the species associated with 4 and 5 phosphates on Hex_5 Hep_3 KDO_2 $GlcN_2$. The polysaccharide portion of LPS also revealed species which corresponded to Hex_5 Hep_3 KDO associated with 2-4 phosphates and an ethanolamine. The present method was proved to be useful to investigate the structural diversity of enterobacterial LPS.

1-1. Introduction

Mass spectrometry is excellent in order to detect many complexed species in a very small quantity. In the field of analysis of LPS, it was no exception, however a main effort was focused onto the characterization of polysaccharide backbone. Analysis of non-stoichiometric substitutions has not been done. Rather, non-stoichiometric substitutions were removed in the early stage of analysis by using HF, and then the purified polysaccharides were almost intensively analyzed. Thus, there have been a few examples providing the information about non-stoichiometric substitutions [19-26]. The LPS of *E. coli* C is known to have a structure of *E. coli* R1 type core polysaccharide. The authors tried to synthesize the hexose region of LPS outer core in order to investigate the molecular recognition of bacteriophage ϕX174 to the *E. coli* C strain as a native host [The master thesis of the auther at Mie Univ. 1992]. According to the Feige's report [27], the LPS was reported to have at most 7 phosphate residues. Since then there is no data about non-stoichiometric substitutions. The specific interaction of bacteriophage ϕX174 spike proteins, which consist of protrusions on the icosahedral capsid, with the LPS of a host strain, *E. coli* C were reported [14,15]. The affinity for the phage proteins was decreased when the LPS was de-phosphorylated by HF treatment [28]. Thus, additional information is needed in order to identify the contributions of the LPS substituents to the recognition mechanism of bacteriophages. In the present chapter, the diversity of non-stoichiometric substitutions on chemically degraded derivatives of LPS from *E. coli* C was analyzed by electrospray ionization-single quadrupole-mass spectrometry (ESI-Q-MS).

It is expected that MS analysis of LPS and LPS derivatives have low sensitivity. Since many phosphate groups attached on the polysaccharide backbone having many negative charges, it is prevented from LPS vaporization. However, phosphate groups

should be favorable for ionization efficiency of negative mode MS analysis.

Thus, an effort was made to find out the method of raising ionization and vapolization efficiency. The desired ions were detected by using a single quadrupole mass spectrometer where an ion source and a mass detector were directly interfaced in a short physical distance.

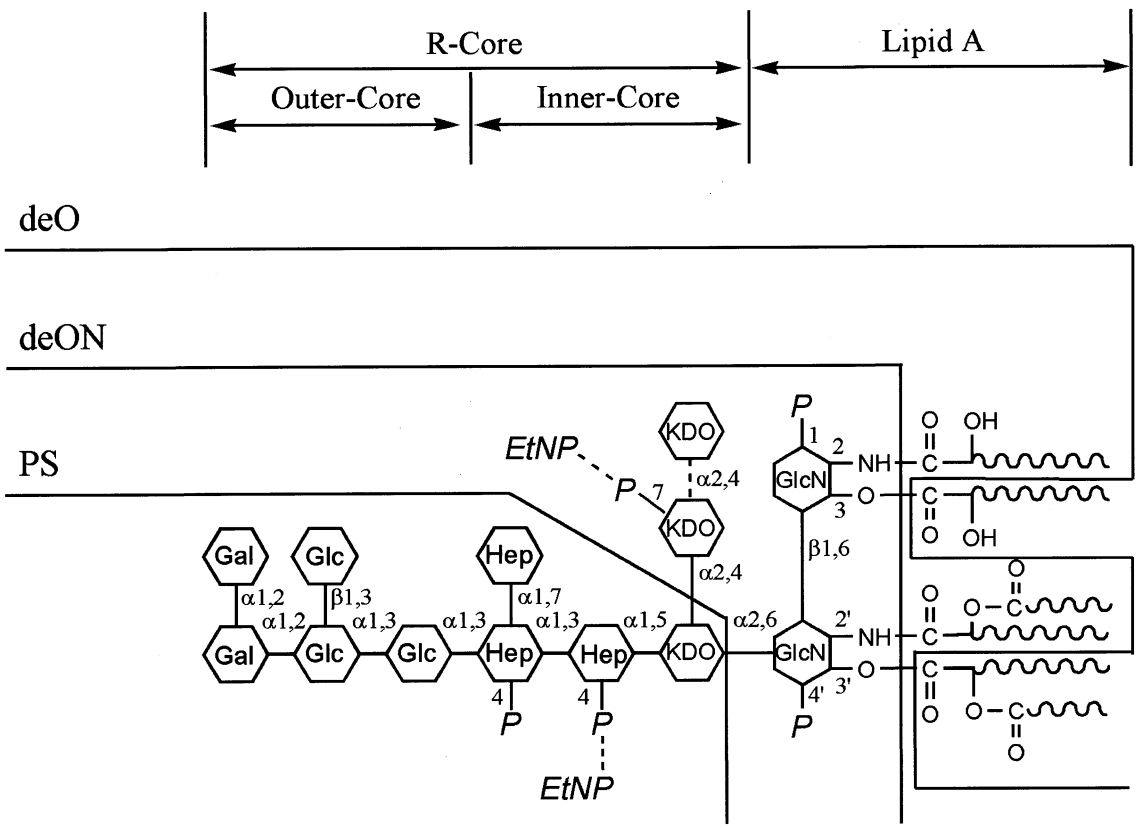


Fig. 1-1. Schematic structure of the LPS of *E. coli* C.

Abbreviations are: Hex, hexose; Hep, L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-D-*manno*-2-octulosonic acid; GlcN, 2-amino-2-deoxy-D-glucose; Gal, D-galactose; Glc, D-glucose; *P*, phosphate; *EtN*, 2-aminoethanol.

1-2. Result and Discussion

1-2.1. Structural diversity of deO derivatives revealed by ESI-Q-MS

The LPS from *E. coli* C was submitted to limited chemical degradations (Fig. 1-1) [14]. The derivatives deO, deON and PS obtained were purified by gel filtration and subjected to analysis by ESI-Q-MS (Table 1-1). The deO showed multiply-charged ions corresponding to $[M-2H]^{2-}$, $[M-3H]^{3-}$, $[M-4H]^{4-}$, and $[M-5H]^{5-}$ (Fig. 1-2). The peaks for $[M-3H]^{3-}$ were intense and well resolved. The deconvolution resulted in three major groups of measured molecular masses differing in the number (0-2) of EtN substituents. Group I consisted of units of Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ with 3-8 residues of phosphate (deO 3P-8P), Group II consisted of units of Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ EtN with 4-8 residues of phosphate (deO+EtN 4P-8P), and Group III consisted of units of Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ EtN_2 with 5-8 residues of phosphate (deO+EtN_2 5P-8P).

The most intense peak in deO was a triply deprotonated anion at *m/z* 1019.6 in Group II which corresponded to a unit of Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ P_5 EtN (deO+EtN 5P). The second most intense peak (79 % to *m/z* 1019.6) was at *m/z* 1046.5 in Group II which corresponded to a unit of Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ P_6 EtN (deO+EtN 6P). Among the Group I units without an EtN moiety, the peaks for deO 4P and deO 5P were the most predominant. Also among the Group III units having two EtN moieties, the peaks for deO+EtN_2 6P and deO+EtN_2 7P were predominant. This indicates that the deO mainly constituted of units with six and seven net negative charges. In addition, the two minor groups of measured molecular mass, which included the KDO III residue, were assigned as units of deO+KDO+EtN 4P and 5P (3281.4 and 3202.8 Da) and deO+KDO+EtN_2 5P and 6P (3404.5 and 3325.4 Da).

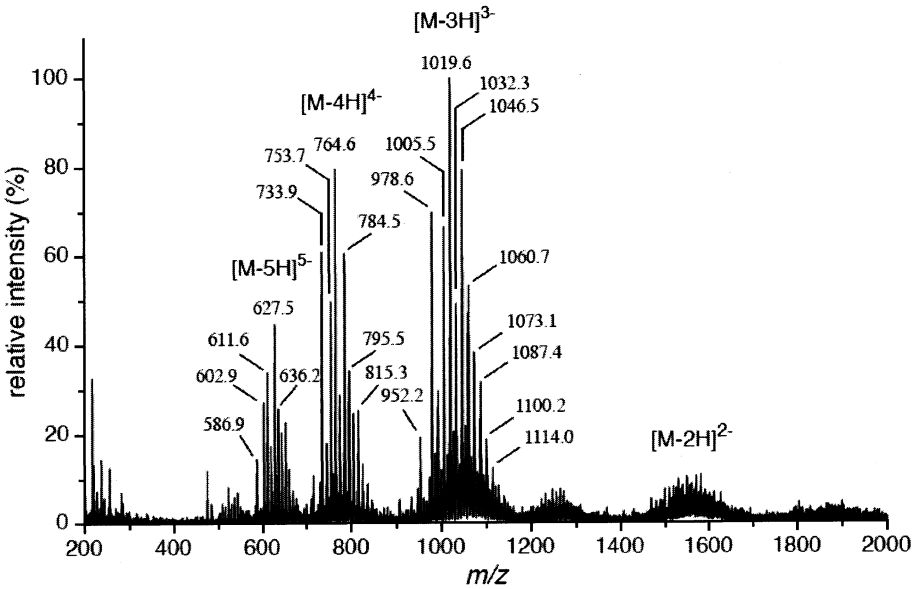


Fig. 1-2. Negative mode ESI-Q-MS spectrum of *O*-deacylated LPS.

1-2.2. Analysis of deON and PS derivatives by anion-exchange HPLC and ESI-Q-MS

Although the deO derivative was only showed a brad and flat peak on the anion exchnge clomatgraphy using a Hi-Trap Q column carring quaternary ammonium group (Fig. 1-3A), the deON was separated into two fractions, deON-A and deON-B, by the anion-exchange chromatography with a sodium chloride gradient (Fig. 1-3B). The purified fractions were dialyzed and subjected to analysis by ESI-Q-MS (Table 1-2).

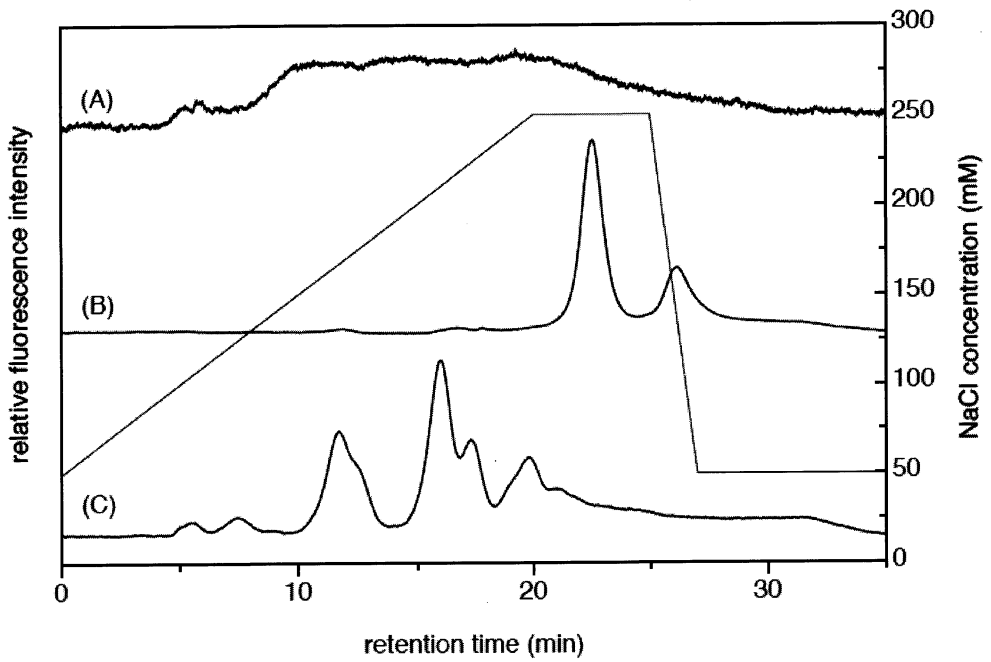


Fig. 1-3. Anion exchange chromatograms of *O*-deacylated LPS (A), *O,N*-deacylated LPS (B) and polysaccharide portion of LPS (C) detected using a fluorescence detector by post-column derivatization using 100 mM taurine and 6 mM sodium periodate (pH 7.0). HPLC conditions were: column, HiTrap Q HP (1 mL); flow rate 1.0 mL/min; eluent, 10 mM sodium phosphate buffer (pH 7.0) with programmed linear gradient 5-25 min with sodium chloride 0-250 mM; Post-column reagent flow rate, 0.3 mL/min; derivatization temperature, 140 °C; fluorescence detection, λ_{ex} 350 nm, λ_{em} 430 nm at 35 °C.

The most intense peaks were a triply deprotonated anion at *m/z* 827.8 corresponding to a unit of Hex_5 Hep_3 KDO_2 $GlcN_2$ P_4 (deON 4P) in deON-A (Fig. 1-4A) and at *m/z* 854.5 corresponding to Hex_5 Hep_3 KDO_2 $GlcN_2$ P_5 (deON 5P) in deON-B (Fig. 1-4B). Thus, the anion-exchange chromatography could differentiate the species within deON by the number of anionic charges. Along with the intense peak of deON 4P in Group I, deON-A showed a peak at *m/z* 868.9 corresponding to the unit of Hex_5 Hep_3 KDO_2 $GlcN_2$ EtN P_5 (deON+EtN 5P) of Group II. These species were the

same in total anionic charges. The molecular mass of 2266.5 Da corresponded to a fragment of 2486.6 Da, which indicated that a unit had undergone the deletion of a KDO residue (deON-KDO 4P).

The deON-B only showed species of Group I without EtN substituent. The predominant species of molecular mass of 2566.7 Da was assigned to a unit of Hex_5 Hep_3 KDO_2 $GlcN_2$ P_5 (deON 5P). The molecular masses of 2486.6 and 2405.7 Da corresponded to the de-phosphorylated fragments (deON 3P and 4P) from the unit of deON 5P. The fragments corresponding to the deletion of a KDO residue were also detected with molecular masses of 2346.2, 2266.8 and 2185.2 Da.

Since the PS showed multiple and overlapping peaks using anion-exchange chromatography (Fig. 1-3C), the PS was analyzed by ESI-Q-MS as a mixture. As shown in Table 1-2 and Fig. 1-4C, the PS showed two main groups of measured molecular mass which were different in the absence (Group I) and the presence (Group II) of the EtN substituent. The molecular mass of 1988.4 Da was assigned to the unit of Hex_5 Hep_3 KDO P_4 EtN (PS+EtN 4P). The de-phosphorylated species were also detected at 1907.6 Da (PS+EtN 3P), 1864.7 Da (PS 3P) and 1785.0 Da (PS 2P). Since the PS derivative was not fractionated by anion-exchange chromatography prior to MS analysis, these de-phosphorylated species would be attributed to the diversity of phosphate substituents rather than the fragmentation during the MS measurement. Moreover, the species which had undergone dehydration such as PS-H_2O with 2-4 phosphates and PS+EtN-H_2O with 3 or 4 phosphates were found in Fig. 1-3C. This phenomenon was specific to PS, which has KDO at the reducing end of the polysaccharide and may be attributed to the formation of an anhydro KDO moiety during the acid hydrolysis [30].

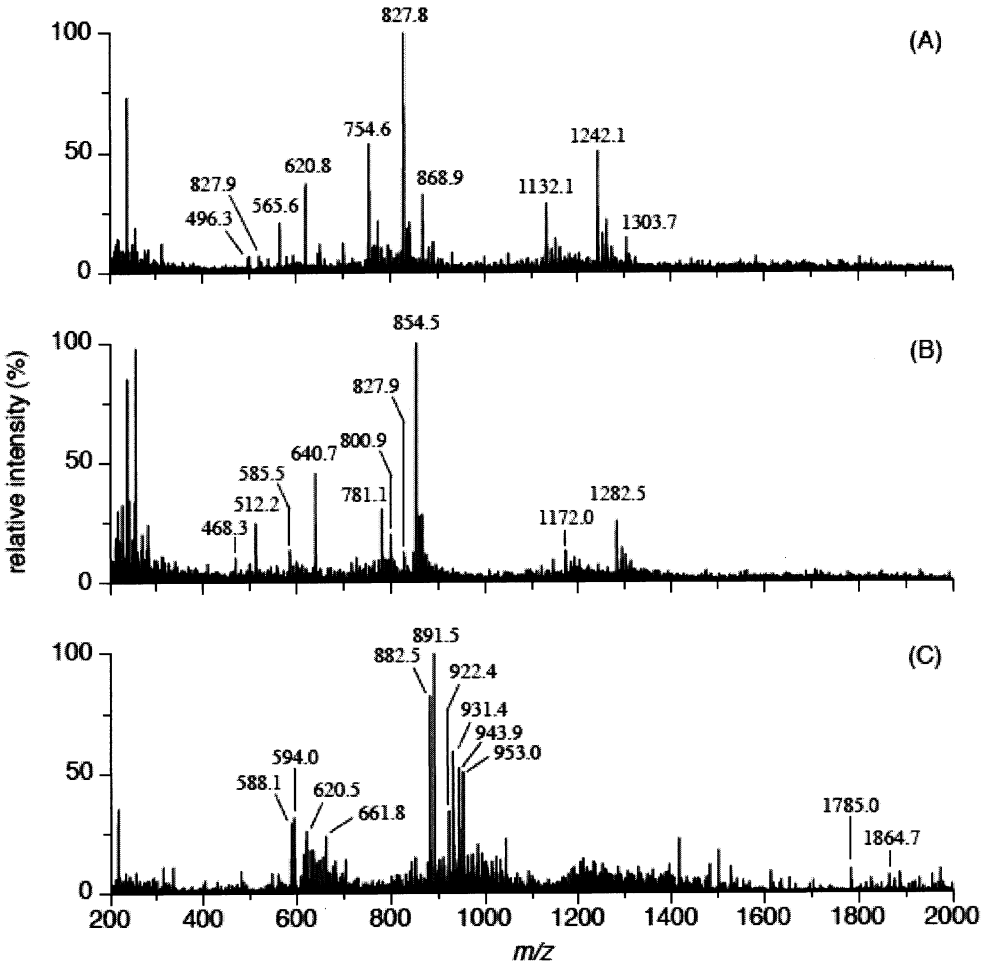


Fig. 1-4. Negative mode ESI-Q-MS spectra of *O,N*-deacylated LPS fraction A (A), fraction B (B), and polysaccharide portion of LPS (C).

Table 1-1. Negative ion ESI-Q-MS data and proposed compositions of deO derivative of *E. coli* C LPS.

Group	Species Name	Observed Ion						Molecular Mass		Proposed Composition
		$[M-5H]^{5-}$ (*m/z*)	Rel Int (%)	$[M-4H]^{4-}$ (*m/z*)	Rel Int (%)	$[M-3H]^{3-}$ (*m/z*)	Rel Int (%)	Obsrvd (Da)	Calcd (Da)	
I	deO 3P			713.8	10	952.2	19	2859.4	2859.0	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_3$
I	deO 4P	586.9	15	733.9	61	978.6	70	2939.3	2939.0	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_4$
I	deO 5P	602.9	27	753.7	50	1005.5	67	3019.3	3018.9	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_5$
I	deO 6P	618.9	17	773.9	29	1032.3	49	3099.7	3098.9	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_6$
I	deO 7P	634.9	22	793.7	31	1058.7	39	3179.1	3178.9	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_7$
I	deO 8P	650.8	12	814.0	16	1085.8	23	3259.7	3258.8	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_8$
II	deO+EtN 4P	595.4	3	744.6	18	992.9	30	2982.0	2982.0	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_4\ EtN$
II	deO+EtN 5P	611.6	34	764.6	80	1019.6	100	3062.4	3062.0	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_5\ EtN$
II	deO+EtN 6P	627.5	45	784.5	61	1046.5	79	3142.3	3141.9	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_6\ EtN$
II	deO+EtN 7P	643.4	20	804.6	25	1073.1	38	3222.2	3221.9	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_7\ EtN$
II	deO+EtN 8P	659.7	12	824.8	13	1100.2	19	3303.4	3301.9	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_8\ EtN$
II	deO+KDO+EtN 4P	640.2	3	800.4	4	1066.6	15	3202.8	3202.1	$Hex_5\ Hep_3\ KDO_3\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_4\ EtN$
II	deO+KDO+EtN 5P	655.3	3	819.0	4	1093.2	11	3281.4	3282.0	$Hex_5\ Hep_3\ KDO_3\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_5\ EtN$
III	deO+EtN_2 5P			775.0	12	1034.0	19	3104.5	3105.0	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_5\ EtN_2$
III	deO+EtN_2 6P	636.1	26	795.3	34	1060.7	53	3185.3	3185.0	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_6\ EtN_2$
III	deO+EtN_2 7P	652.0	23	815.3	25	1087.4	31	3265.1	3265.0	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_7\ EtN_2$
III	deO+EtN_2 8P	668.1	8	835.1	9	1114.0	13	3345.0	3344.9	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_8\ EtN_2$
III	deO+KDO+EtN_2 5P	664.1	1	830.2	3	1107.6	7	3325.4	3325.1	$Hex_5\ Hep_3\ KDO_3\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_5\ EtN_2$
III	deO+KDO+EtN_2 6P	679.8	3	850.3	3	1133.8	4	3404.5	3405.0	$Hex_5\ Hep_3\ KDO_3\ GlcN_2\ C14{:}0(3\text{-}OH)_2\ P_6\ EtN_2$

Abbreviations were: Hex, hexose; Hep, L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-D-*manno*-2-octulosonic acid; GlcN, 2-amino-2-deoxy-D-glucose; C14:0(3-OH), 3-hydroxytetradecanoic acid; P, phosphate; EtN, 2-aminoethanol. 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; GlcN, 161.16; P, 79.98; EtN, 43.04; C14:0(3-OH), 226.36; C14:0, 210.36; C12:0, 182.31 Da.

Table 1-2. Negative ion ESI-Q-MS data and proposed compositions of deON-A, deON-B and PS derivatives of *E. coli* C LPS.

Sample	Group	Species Name	Observed Ion								Molecular Mass		Proposed Composition
			$[M-5H]^{5-}$ (*m/z*)	Rel Int (%)	$[M-4H]^{4-}$ (*m/z*)	Rel Int (%)	$[M-3H]^{3-}$ (*m/z*)	Rel Int (%)	$[M-2H]^{2-}$ (*m/z*)	Rel Int (%)	Obsrvd (Da)	Calcd (Da)	
deON-A													
	I	deON 4P	496.3	6	620.8	38	827.8	100	1242.1	51	2486.6	2486.6	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ P_4$
	I	deON-KDO 4P			565.6	21	754.6	54	1132.2	29	2266.5	2266.5	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ P_4$
	II	deON+EtN 5P	521.1	7	651.5	12	868.9	33	1303.7	15	2609.9	2609.6	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ P_5\ EtN$
deON-B													
	I	deON 3P					800.9	20			2405.7	2406.6	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ P_3$
	I	deON 4P					827.9	13			2486.6	2486.6	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ P_4$
	I	deON 5P	512.3	25	640.7	46	854.5	100	1282.5	26	2566.7	2566.5	$Hex_5\ Hep_3\ KDO_2\ GlcN_2\ P_5$
	I	deON-KDO 3P					727.4	11			2185.2	2186.6	$Hex_5\ Hep_3\ KDO\ GlcN_2\ P_3$
	I	deON-KDO 4P					754.6	7			2266.8	2266.5	$Hex_5\ Hep_3\ KDO\ GlcN_2\ P_4$
	I	deON-KDO 5P	468.3	11	585.5	13	781.1	31	1172.0	14	2346.2	2346.5	$Hex_5\ Hep_3\ KDO\ GlcN_2\ P_5$
PS													
	I	PS 2P					594.0	32	891.5	100	1785.0	1784.5	$Hex_5\ Hep_3\ KDO\ P_2$
	I	PS 3P					620.5	22	931.4	59	1864.7	1864.4	$Hex_5\ Hep_3\ KDO\ P_3$
	I	PS 4P					647.2	13			1944.6	1944.4	$Hex_5\ Hep_3\ KDO\ P_4$
	I	PS-H_2O 2P					588.1	29	882.5	82	1767.2	1766.4	$Hex_5\ Hep_3\ KDO\ P_2\ -H_2O$
	I	PS-H_2O 3P					614.8	16	922.4	32	1847.1	1846.4	$Hex_5\ Hep_3\ KDO\ P_3\ -H_2O$
	I	PS-H_2O 4P					641.3	13			1926.9	1926.4	$Hex_5\ Hep_3\ KDO\ P_4\ -H_2O$
	II	PS+EtN 3P					634.7	15	953.0	51	1907.6	1907.5	$Hex_5\ Hep_3\ KDO\ P_3\ EtN$
	II	PS+EtN 4P					661.8	24			1988.4	1987.4	$Hex_5\ Hep_3\ KDO\ P_4\ EtN$
	II	PS+EtN-H_2O 3P					629.0	18	943.9	52	1889.9	1889.5	$Hex_5\ Hep_3\ KDO\ P_3\ EtN\ -H_2O$
	II	PS+EtN-H_2O 4P					655.6	15			1969.8	1969.4	$Hex_5\ Hep_3\ KDO\ P_4\ EtN\ -H_2O$

Abbreviations were: Hex, hexose; Hep, L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-D-*manno*-2-octulosonic acid; GlcN, 2-amino-2-deoxy-D-glucose; P, phosphate; EtN, 2-aminoethanol. 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; GlcN, 161.16; P, 79.98; EtN, 43.04; C14:0(3-OH), 226.36; C14:0, 210.36; C12:0, 182.31 Da.

1-3. Experimental

1-3.1. LPS Preparation and limited chemical degradations

The LPS of *E. coli* C (NBRC 13898) was extracted from the cultured cells [16] using a phenol–chloroform–petroleum ether method [17]. The extracted LPS was submitted to limited chemical degradations [14] according to the reported method with anhydrous hydrazine (NH_2NH_2) at 37°C for 30 min to form *O*-deacylated LPS (deO) and then further treated with 4 M KOH at 125°C for 18 h to form *O,N*-deacylated LPS (deON). The polysaccharide part of LPS (PS) was obtained by hydrolyzing the LPS in 1 % acetic acid at 100°C for 2 h. The degraded LPS derivatives were purified by gel filtration through a Bio Gel P4 column (1.5 cm I.D. × 110 cm) (Bio-Rad, Hercules, CA, USA) eluted with pyridine/acetic acid/H_2O (10:5:1000 v/v/v at pH 4.2). The saccharide-containing fractions were detected using a phenol-H_2SO_4 method [29] or flow injection analysis in line with a HPLC fluorescence detector after derivatization using taurine and sodium periodate [18] and then they were combined and lyophilized.

1-3.2. ESI-Q-MS

ESI-Q-MS was performed in the negative ion mode using a Waters Micromass ZQ 2000 system (Waters, USA). Samples (0.1 mg/mL) were dissolved in a mixture of 50/50 (v/v) methanol and 10 mM ammonium acetate (pH 6.8), and sprayed at a flow rate of 10 μL/min. The capillary entrance voltage was set to 3.0 kV, the dry gas temperature to 150°C, and the cone voltage from -10 to -60 V. The mass range was set from 200 to 2000 *m/z*.

1-3.3. HPLC analysis of LPS with post-column derivatization

HPLC experiments were performed on an LC-10ADVP system with three pumps, a fluorescence detector, and a hot air oven (Shimadzu, Kyoto, Japan). The LPS derivatives were separated at 35°C on a HiTrap Q HP column (1 mL) (GE Healthcare Biosciences, Tokyo, Japan) by elution with 10 mM sodium phosphate (pH 7.4) (flow rate 1.0 mL/min) containing up to 250 mM sodium chloride delivered by two pumps. The third pump supplied the post-column reagent (0.3 mL/min) containing 100 mM taurine and 6 mM sodium periodate (pH 7.0). The mixture was heated through a 0.5 mm I.D. × 20 m stainless coil at 140°C, and cooled through a 0.5 mm I.D. × 10 m stainless coil in the column oven to 35°C. The detection was performed λ_{ex} at 350 nm and λ_{em} at 430 nm.

Chapter 2

Separation and characterization of lipopolysaccharide related compounds by HPLC/post-column fluorescence derivatization (HPLC/FLD) and capillary zone electrophoresis/mass spectrometry (CZE/MS)

Abstract

The *O,N*-deacylated derivative (deON) and polysaccharide part (PS) from the lipopolysaccharide (LPS) of *Escherichia coli* C strain were separated by strongly basic anion-exchange chromatography (SAX) based on the differences in the number of charged phosphate and ethanolamine substituents. They were also successfully separated and characterized by capillary zone electrophoresis and subsequent ESI-ion trap-MS (CZE/ESI-IT-MS). The *O*-deacylated LPS (deO) presented as a broad peak in CZE/ESI-IT-MS. However, more than twelve species could be discriminated by an extracted ion electropherogram (EIE) and monitoring the species which have different numbers of phosphate and ethanolamine substituents on polysaccharide backbone.

2-1. Introduction

In the preceding chapter, the author revealed the diversity of non-stoichiometric substituents on the inner R-core of *E. coli* C LPS by ESI-Q-MS analysis using deacylated derivatives [31]. The LPS was found to have diverse substitution with up to eight phosphate, three KDO, and two ethanolamine residues on the polysaccharide backbone [Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$]. In the present chapter, the separation of the deacylated derivatives of the LPS based on the variation in the charged

substituents was investigated by strongly basic anion-exchange chromatography (SAX-HPLC). This gave superior separation and simultaneous characterization by the capillary zone electrophoresis coupled with electrospray ionization-ion trap-mass spectrometry (CZE/ESI-ITMS).

2-2. Results and discussion

2-2.1. Separation of LPS related compounds by strongly basic anion-exchange chromatography based on the numbers of negatively charged residues

The separation and characterization of the LPS of *E. coli* C strain and its related compounds, such as *O*-deacylated (deO), *O,N*-deacylated (deON) LPSs, and polysaccharide part of LPS (PS) has proved challenging. The derivatives are not retained on reversed phase silica gel columns having C18, amino, cyano or phenyl groups. They also bind irreversibly to anion-exchange columns having diethylaminoethyl groups on dextran or non-porous resins because the derivatives have polyvalent substitution with negatively charged phosphate and positively charged ethanolamine residues. On the other hand, a strongly basic anion-exchange column with quaternary ammonium group (HiTrap Q HP) was found to be suitable for the retention of deON and PS derivatives. The analytes could then be eluted by an increasing sodium chloride gradient (Fig. 2-1A and 2-1B). The deO derivative was not eluted out even when high concentrations of NaCl were applied to the column (Fig. 2-1C).

The LPS related compounds do not have any useful chromophores, thus the compounds were separated and detected in the HPLC system combined with a method for post-column derivatization using taurine and sodium periodate [32]. This method is effective for compounds having a *vicinal*-diol structure, which is cleaved oxidatively to

yield a pair of aldehydes by periodate. This then reacts with taurine to form adducts which exhibit fluorescence [18]. As a preliminary experiment, an LPS sample was injected to the HPLC system without a column (flow injection analysis), mixed with the taurine and sodium periodate at 130 °C, and detected fluorometrically (λ_{ex} = 350, λ_{em} = 430 nm). LPS in the range of 0.31–10 μg/10 μL was precisely detected by the method. The linearity of the calibration curves was good ($r^2 > 0.999$) and the limit of detection was ca. 0.1 μg/10 μL.

The two major peaks from the deON derivative (Fig. 2-1A) were fractionated and analyzed by the ESI-Q-MS after the equilibrium dialysis [31]. The fractions were characterized as deON 4P and 5P [Hex_5 Hep_3 KDO_2 $GlcN_2$ P_4 and P_5] by the negative ion mode of ESI-Q-MS (*m/z* at 827.8 for deON 4P and at *m/z* 854.5 for deON 5P as $[M-3H]^{3-}$ ions). The former fraction contained a small amount of deON+ EtN 5P [Hex_5 Hep_3 KDO_2 $GlcN_2$ EtN P_5] (*m/z* at 868.9). The strongly basic anion-exchange (SAX) column (HiTrap Q HP) separated the deON derivatives based on the differences in the numbers of negatively charged phosphate substituents.

The PS derivative was separated into multiple peaks as shown in Fig. 2-1B. Although the deON and PS derivatives were derived from the same LPS preparation, the many species corresponding to phosphate and ethanolamine residues were detected more precisely from the PS derivative. This is because the conditions for the PS preparation (1% acetic acid, 100 °C, for 2 h) were milder than that of the deON (4M KOH, 125 °C, for 18 h). In the previous chapter, the ESI-Q-MS of the PS derivative revealed that the derivative contained PS 2P [Hex_5 Hep_3 KDO P_2] (*m/z* at 891.5) and PS + EtN 3P [Hex_5 Hep_3 KDO EtN P_3] (*m/z* at 953.0) as the major species detected as $[M-2H]^{2-}$ ions. Thus the two intense peaks in Fig. 2-1B correspond to the two species above. The other overlapping peaks were not characterized.

On the other hand, the deO derivative, which is corresponded to Hex_5 Hep_3

KDO_2 $GlcN_2$ C14:0(3–$OH)_2$ with various numbers of negatively charged substituents, was not eluted from the HiTrap Q HP column even when amobile phase containing 250 mM NaCl was applied.

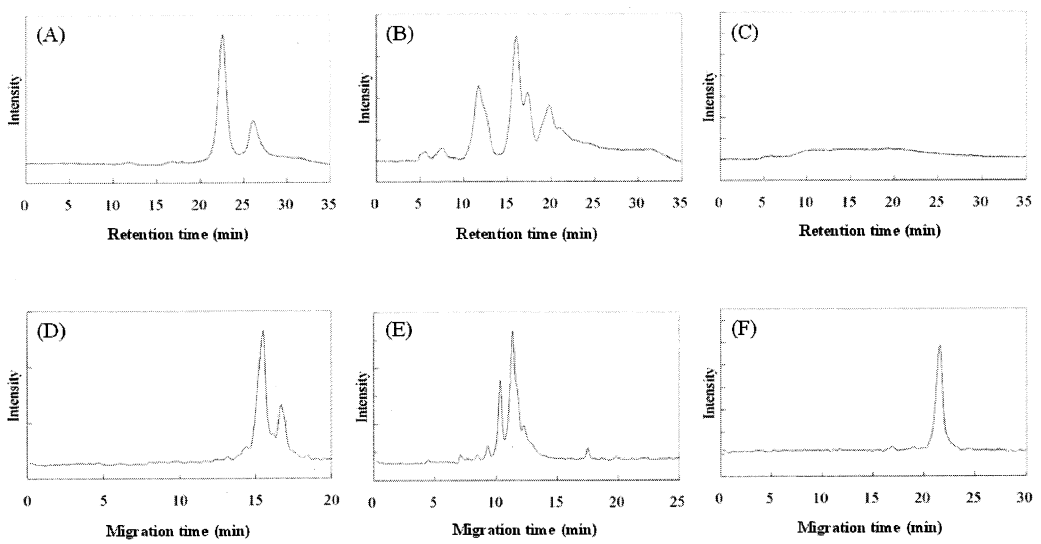


Fig. 2-1. Concurrent separation of *O,N*-deacylated LPS (A and D), polysaccharide part of LPS (B and E), and *O*-deacylated LPS (C and F) by strongly basic anion-exchange chromatography/post-column fluorometric derivatization (SAX-FLD) (upper) and capillary zone electrophoresis/electrospray ionization-ion trap-mass spectrometry (CZE/ESIIT-MS) (lower).

The HPLC conditions were: column, HiTrap Q HP (1.0 mL); flow rate, 1.0 mL/min; eluent, 10 mM sodium phosphate (pH 7.0) with programmed linear gradient 5–25 min with sodium chloride 0–250 mM; post-column reagent, 100 mM taurine and 6 mM sodium periodate (pH 7.0); post-column reagent flow rate, 0.3 mL/min, fluorescence detection, λ_{ex} 350 nm and λ_{em} 430 nm. The CZE conditions were: capillary, bare fused-silica capillary (50 μm ID × 100 cm); running electrolyte, 10 mM ammonium acetate (pH 9.0) (D and E) and 50 mM ammonium acetate (pH 9.0) (F); applied voltage, 30 kV (D and E) and 10 kV (F) The ESI-IT-MS conditions were: detection at negative ion mode; sheath solutions, 50% (v/v) aqueous methanol (D and E) and 0.1% (v/v) formic acid in 50% (v/v) aqueous methanol; flow rate, 5 μL/min; scanning mass range, 500–1500 *m/z*.

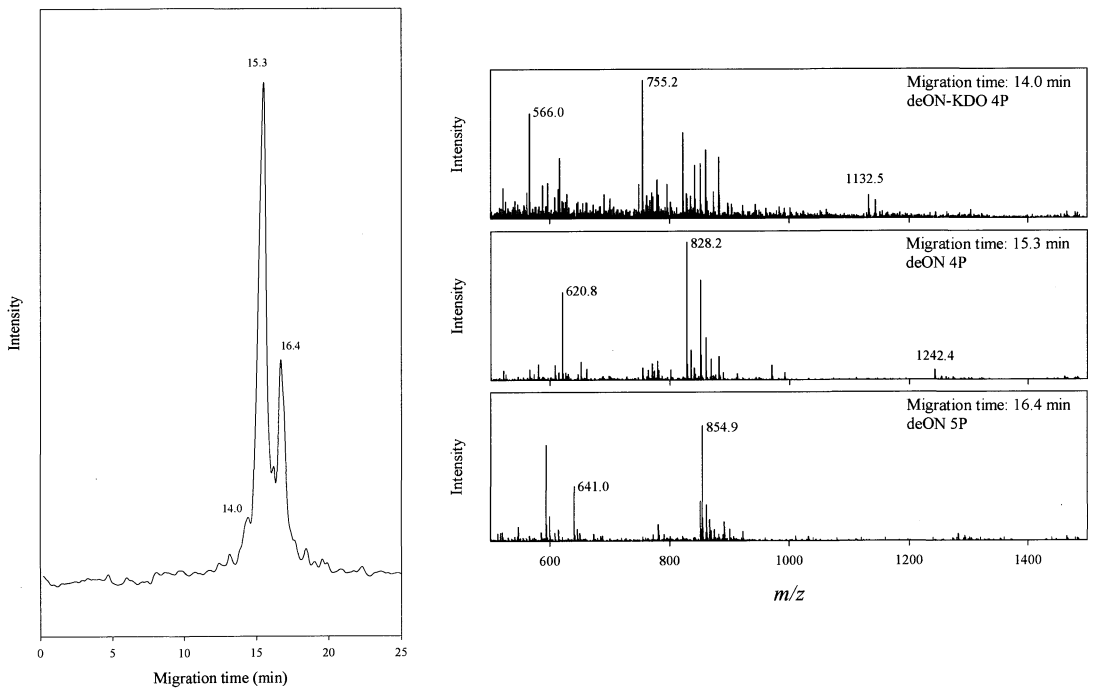


Fig. 2-2. Total ion electropherogram of CZE/ESI-IT-MS of *O,N*-deacylated LPS (deON) and extracted mass spectra at 14.0, 15.3 and 16.4 min.

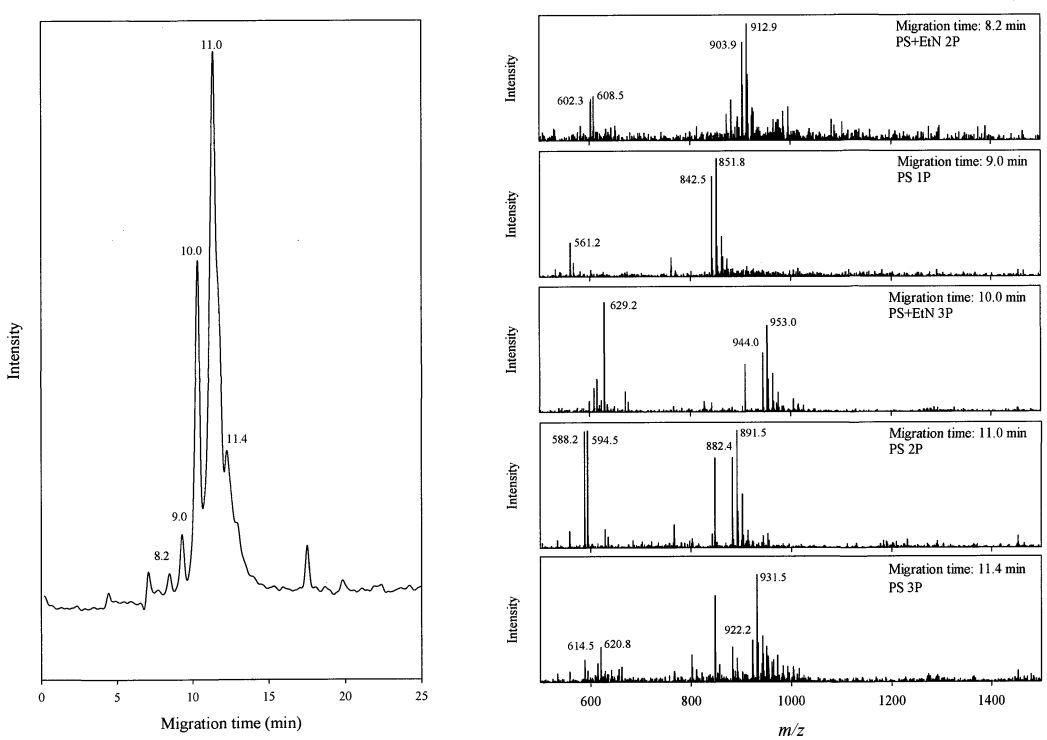


Fig. 2-3. Total ion electropherogram of CZE/ESI-IT-MS of polysaccharide part of LPS (PS) and extracted mass spectra at 8.2, 9.0, 10.0, and 11.0 min.

2-2.2. Separation and characterization of deON and PS derivatives by capillary zone electrophoresis and subsequent electrospray ionization-ion trap-mass spectrometry (CZE/ESI-IT-MS)

The deON and PS derivatives were separated by SAX-HPLC, however, direct injection of the eluted fractions into a mass spectrometer was failed for ESI-MS and matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS) because of the high salt concentration in the elution buffer. Thus capillary zone electrophoresis/mass spectrometry (CZE/MS) was investigated as an alternative method. CZE/MS has been previously reported for the analyses of the deacylated derivatives of LPS from *Haemophilus influenzae* [33, 34].

A fundamental mechanism of CE operation is electro-osmotic flow (EOF). EOF is a bulk flow of liquid in the capillary and is a consequence of the charge on the interior surface of the capillary wall. EOF causes movement of almost all species (cation, anion, and non-ion), regardless of their charge, in the same direction. Therefore, it is not necessary to use a highly concentrated buffer such as 250 mM NaCl as in SAX-HPLC. Because a fused-silica capillary can also bear anionic charge ($Si–O^-$), the LPS related compounds are not adsorbed onto the capillary. Consequently, running buffers with a concentration from 10 to 50 mM can be used to control the solute–capillary wall interactions during the analysis. As a volatile running buffer, ammonium formate and acetate were thought to be compatible with the ion trap-mass spectrometry (IT-MS) analysis of the fractions directly from the capillary. Although morpholine buffer has been reported as appropriate in some papers [4, 5], 10mM ammonium formate and ammonium acetate had superior sensitivities at the IT-MS detector.

A buffer of 10 mM ammonium acetate (pH 9.0) was used as running electrolyte,

and the electrophoresis was carried out at 30 kV. These conditions afforded good separation efficiencies for the deON and PS derivatives. However, it did not separate the more challenging deO derivative which consisted of more than twenty components. The concentration of the running electrolyte was thus changed to 50 mM to improve the separation for the deO derivative. This also causes an increase in the sensitivity of the IT-MS by a stacking effect between the sample dissolved in pure water and 50mM running electrolyte. However, the electrophoresis was carried out at 10 kV to keep the current below 15 mA. The current was the upper limit for reproducible results when using an air circle cooling device.

The sheath solution is one of the most important factors in the sensitivity of CE/MS analysis. The composition and flow rates of the sheath solution were optimized by trial experiments using deON and deO derivatives. Aqueous 50% methanol including 5–10 mM ammonium formate, was found to be suitable in the previous study for the ESI-Q-MS analyses of the LPS related compounds, but here resulted in peak disappearance for the deON and deO derivatives. This could be due to ion suppression by charge neutralization with the ammonium ion. Consequently, an aqueous 50% methanol solution without buffer salt was selected for the experiment. The deON and PS derivatives mainly generated trivalent negative ions $[M-3H]^{3-}$. However, tetravalent $[M-4H]^{4-}$ and pentavalent $[M-5H]^{5-}$ ions were generated in the case of the deO derivative, where many negatively charged phosphates are attached. Addition of 0.1% formic acid into aqueous 50% methanol showed improved sensitivity at the mass spectrometer for deO derivative by changing the ionized state of quadruply negative $[M-4H]^{4-}$ ions to triply negative $[M-3H]^{3-}$ ions and thereby the intensity at the IT-MS was dramatically increased. Controlling the pH of the sheath solution resulted in increasing the intensity of observed ions. In addition, the flow rate of the sheath solution was optimized at 5 μL/min in order to increase the efficiency of the electrospray.

The total ion electropherogram (TIE) of CZE/ESI-IT-MS of the deON derivative is shown in Fig. 2-1D and are compared with the SAX-HPLC/FLD chromatogram (Fig. 2-1A). This is the optimized result for the analysis of the deON derivative by CZE using a volatile buffer salt instead of NaCl in the SAX-HPLC. The mass spectra at each of the peaks for the TIE are summarized in Fig. 2-2. The two intense peaks with the migration times (Mt) of 15.3 and 16.4 min were characterized as the species deON 4P with an m/z = 828.2 for $[M-3H]^{3-}$ and 1242.4 for $[M-2H]^{2-}$ and deON 5P with an m/z = 854.9 for $[M-3H]^{3-}$. In addition to the two peaks, a shoulder peak at 14.0 min was also characterized as deON-KDO 4P with an m/z = 755.2 for $[M-3H]^{3-}$ and 1132.5 for $[M-2H]^{2-}$.

Fig. 2-1 is the TIE of CZE/ESI-IT-MS of the PS derivative, wherethe shape of the chromatogram was consistent with the SAXHPLC/FLD chromatogram (Fig. 2-1B). The mass spectra extracted at the peaks of the TIE are also summarized in Fig. 2-3. The peak at 10.0 min was characterized as the species of PS + EtN 3P with an m/z = 953.0 for $[M-2H]^{2-}$, and the most intense peak, at 11.0 min, was PS 2P with an m/z = 891.5. As minor components, PS 3P (m/z = 931.5) at 11.4 min, PS 1P (m/z = 851.8) at 9.0 min, and PS + EtN 2P (m/z = 912.9) at 8.2 min were also detected. Although PS + EtN 2P has the same total charge with PS 1P, due to the negatively charged phosphates being partly neutralizing by the positively charged ethanolamine substituents, the migration of PS + EtN 2P (8.2 min) tends to be faster than that of PS 1P (9.0 min). This was also true for the case for PS + EtN 3P (10.0 min) and PS 2P (11.0 min).

2-2.3. Characterization of deO derivative by CZE/ESI-IT-MS and further extracted ion electropherograms (EIE)

The TIE of CZE/ESI-IT-MS of the deO derivative only showed a broad and

poorly resolved peak (Fig. 2-1F). In the previous chapter, the ESI-Q-MS revealed that the deO derivative consisted of multiple species corresponding to diverse substitution at up to 8 phosphates, two ethanolamines, and an additional third KDO residue on the deO backbone of Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$. As the total number of charged groups increased, electrophoretic resolution based on the numbers of charged residues declined. The hydrophobic fatty acid chains on deO also interfered with the separation because of the formation of aggregates (e.g. micelles). However, the species in the deO derivative were reasonably discriminated by the extracted ion electropherograms (EIEs) (Fig. 2-4). The EIEs were drawn by monitoring the individual ions for triply deprotonated ions $[M-3H]^{3-}$ corresponding to species having 3–8 phosphate and 0–2 ethanolamine residues on deO backbone [Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$].

The species with more total charges tended to have larger migration times. The relative intensities of the EIEs and proposed composition of the species having total charges from −5 to −8 are summarized in Table 2-1. The main species in the deO derivative were estimated to be deO + EtN 6P (*m/z* = 1046.4), deO + EtN 5P (*m/z* = 1019.7), deO 5P (*m/z* = 1005.4), deO 4P (*m/z* = 978.8), deO + EtN_2 6P (*m/z* = 1060.7), and deO + EtN_2 7P (*m/z* = 1087.3). Fragmentation is not common in ion trap-mass spectrometry because of the mild ionization conditions. Thus the multiple peaks detected on the EIE for the same *m/z* number indicated that there were many different combinations in the pattern of phosphate substitution on the deO backbone. The LPS preparation extracted from the cultured *E. coli* C cell was found to contain 4.9 residues of phosphate on average by ICP-MS experiment (Data not shown). This agrees with the present CZE/ESI-IT-MS analysis of the deO derivative.

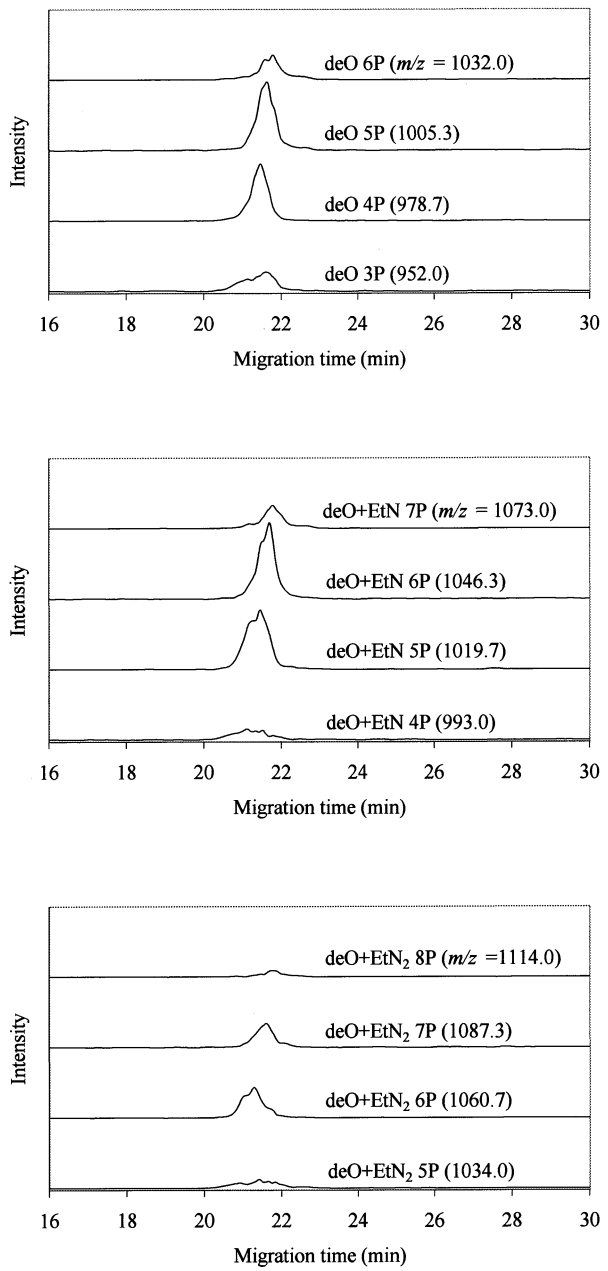


Fig. 2-4. Extracted ion electropherograms (EIEs) of CZE/ESI-IT-MS of *O*-deacylated LPS (deO).

EIEs were drawn for the selected *m/z* values with ±0.5 amu corresponding to the species of various numbers of phosphate and ethanolamine substituents on deO backbone. The series of EIEs were summarized for the species containing no (upper), one (middle), and two (lower) EtN.

Table 2-1. CZE/ESI-IT-MS data of the *O*-deacylated LPS (deO) derived from the *E. coli* C LPS.

Species	Mt	Observed ion		Molecular mass		Formal charge				Proposed composition
	(min)	$[M-3H]^{3-}$	Rel. Int.	Observed	Calculated	KDO	P	EtN	Total	
		(*m/z*)	(%)	(Da)	(Da)					
deO+EtN_2 5P	20.9	1033.9	7	3104.7	3105.0	-2	-5	+2	-5	Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ EtN_2 P_5
deO 3P	21.1	952.4	16	2860.2	2859.0	-2	-3	+0	-5	Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ P_3
deO+EtN 4P	21.1	993.2	14	2982.6	2982.0	-2	-4	+1	-5	Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ EtN P_4
deO+EtN_2 6P	21.3	1060.7	40	3185.1	3185.0	-2	-6	+2	-6	Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ EtN_2 P_6
deO 4P	21.4	978.8	75	2939.4	2939.0	-2	-4	+0	-6	Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ P_4
deO+EtN 5P	21.4	1019.7	78	3062.1	3062.0	-2	-5	+1	-6	Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ EtN P_5
deO 5P	21.6	1005.4	90	3019.2	3018.9	-2	-5	+0	-7	Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ P_5
deO+EtN_2 7P	21.6	1087.3	31	3264.9	3265.0	-2	-7	+2	-7	Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ EtN_2 P_7
deO+EtN 6P	21.7	1046.4	100	3142.2	3141.9	-2	-6	+1	-7	Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ EtN P_6
deO 6P	21.8	1032.0	31	3099.0	3098.9	-2	-6	+0	-8	Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$
P_6deO+EtN 7P	21.8	1073.0	30	3222.0	3221.9	-2	-7	+1	-8	Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ EtN P_7
deO+EtN_2 8P	21.8	1114.0	8	3345.0	3344.9	-2	-8	+2	-8	Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$ EtN_2 P_8

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; GlcN, 161.16; P, 79.98; EtN, 43.04; C14:0(3-OH), 226.36 Da.

2-3. Experimental

2-3.1. Materials

Fused-silica capillaries with 0.375 mm OD × 0.050 mm ID were obtained from GL Sciences Inc. (Tokyo, Japan). HiTrap Q HP column (1 mL) was obtained from GE Healthcare Bio-Sciences (Tokyo, Japan). Methanol, 2-propanol, acetonitrile, ammonium acetate, ammonium formate, acetic acid, formic acid, 28% ammonia solution and morpholine 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). Purified water was produced by Milli-Q system from Nihon Millipore (Tokyo, Japan).

2-3.2. Samples

2-3.2.1. Lipopolysaccharide of *E. coli* C

The lipopolysaccharide (LPS) of *E. coli* C was extracted from cultured cells [16] by the phenol–chloroform–petroleum ether method [17].

2-3.2.2. Deacylated derivatives of LPS

The LPS of *E. coli* C was treated with anhydrous hydrazine (NH_2NH_2) at 37 °C for 30 min [14, 35] to form *O*-deacylated LPS (deO) and then further treated with 4 M KOH at 125 ˚C for 18 h [36] to form *O,N*-deacylated LPS (deON). The polysaccharide (PS) part of LPS was obtained by hydrolyzing the LPS in 1% acetic acid at 100 °C for 2

h [14, 37]. These degraded LPS derivatives were purified by gel filtration through Bio Gel P4 in a 1.5 cm ID × 110 cm length column (Bio-Rad, Hercules, CA, USA) eluted with a pyridine/acetic acid/H_2O ratio of 10:5:1000 (v/v/v) at pH 4.2. The saccharide-containing fractions were detected by the phenol–H_2SO_4 method [29] or flow injection analysis using an inline HPLC fluorescence detector after derivatization using taurine and sodium periodate [32]. These fractions were then combined and lyophilized.

2-3.3. Methods

2-3.3.1. HPLC/post-column fluorescence derivatization (HPLC/FLD)

All HPLC experiments were carried out on an LC-10ADVP 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 one was used to deliver 10 mM sodium phosphate buffer (pH 7.4) and pump two was used to deliver 10 mM sodium phosphate buffer (pH 7.4) containing 250 mM NaCl. The mobile phase was composed of the mixture of pump one and two. The gradient was delivered at 1 mL/min as follows: 0–5 min, 0% pump two; 5–20 min, 0–100% pump two; 20–25 min, 100% pump two; 25–27min, 100–0% pump two; 27–35 min, 0% pump two; giving a total loop time of 35 min. Pump three was used to deliver the post-column reagent at 0.3 mL/min, which was prepared with 12.5 g of taurine and 1.28 g of sodium periodate in 1000 mL of purified water. The 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 length and 0.5 mm ID × 10 m length, respectively. The detection was performed at an excitation wavelength of 350 nm and an emission wavelength of 430 nm.

2-3.3.2. Capillary zone electrophoresis (CZE)

Capillary zone electrophoresis was performed using an Agilent 3D-CE system (Agilent Technologies, Tokyo, Japan). All aqueous solutions were filtered through a 0.45 μm filter (Nihon Millipore, Tokyo, Japan) before use. The separation was achieved over a ca. 100 cm length of bare fused-silica capillary. The inlet and outlet of the capillary were made with straight cuts using a SGT capillary column cutter with a rotating diamond blade (Scientific Glass Technology BV, Middelburg, Netherlands). The polyimide coating on both ends (each 5.0 mm) of the capillary was peeled off using a lighter and wiped with methanol. Ammonium acetate was selected as the volatile running electrolyte in all the experiments. The software used was 3D-CE Chem Station Rev. A 09.03 (Agilent Technologies, Inc., USA).

2-3.3.3. Electrospray ionization-ion trap-mass spectrometry (ESI-IT-MS)

Mass spectra were acquired using an Agilent Ion Trap-Mass Spectrometer (Agilent Technologies, Inc., USA), with scanning resolution of 13,000 *m/z/s* in the standard scanning range of 200–2200 *m/z*. The electrospray interface was set in negative ionization mode with the capillary voltage set at 3.5 kV and the heat source at 300 °C. Nitrogen was used as a drying (10 L/min) and nebulizing gas (2.5–10 psi). The software versions used were MSD trap control version 5.1 and Data Analysis version 2.2 (Agilent

Technologies, Inc., USA).

2-3.3.4. Capillary zone electrophoresis/electrospray ionization-ion trap-mass spectrometry (CZE/ESI-IT-MS)

Agilent Standard Interface; including the gas-junction, liquid junction, and coaxial liquid sheath-flow were used for coupling 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 deON and PS derivatives. 50 mM ammonium acetate (pH 9.0) was used as the running electrolytes for deO derivative and the electrophoresis was carried out at 10 kV to keep the current below 15mA. In the CZE/ESI-IT-MS, ca. 10 nL of the sample was typically injected at 50 mbar for a duration of 10 s.

The composition and flow rate of the sheath solution were optimized by trial experiments using deON and deO derivatives. Aqueous 50% methanol was suitable for the analyses of deON and PS, but for deO derivatives, aqueous 50% methanol including 0.1% formic acid showed an improved sensitivity at the mass spectrometer. After the investigation on the effect of flow rate (ranged from 2 to 10 μL/min), the flow rate was optimized at 5 μL/min in order to increase the sensitivity and efficiency of the electrospray.

Chapter 3

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

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), using tributylamine as an ion-pair reagent. In the conditions used in the present chapter, 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 characterization of the species corresponding to the diverse non-stoichiometric substitutions in LPS related compounds.

3-1. Introduction

In the preceding chapter, the author has successfully demonstrated electrospray ionization-mass spectrometry (ESI-MS) can determine the diversity of non-stoichiometric substitutions on LPS related compounds, such as deON, PS and deO, derived from the limited chemical degradation of the LPS [31]. The multiple species were detected for deON and PS derivatives by use of strong 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) [38].

When the strongly basic anion exchange (SAX) HPLC has been used to analyze and fractionate deON and PS derivatives in the past, it has been difficult to interface 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 [39]. This approach has been applied to the separation and characterization of oligosaccharides derived from hyaluronic acid [40] and of highly sulfated species from heparan sulfate [41] and heparin [42]. The RPIP-HPLC method should be applicable to the separation and characterization of the LPS related compounds. In the present chapter, the author

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.

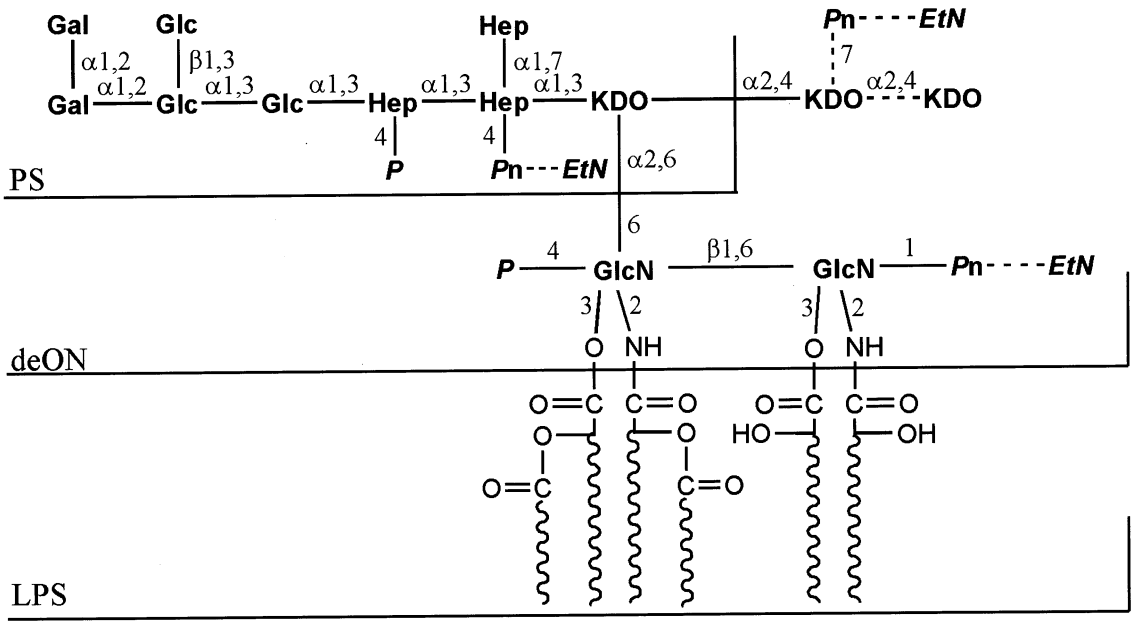


Fig. 3-1. Chemical structure of LPS of *E. coli* C strain and the positions of the limited chemical degradations to afford the *O,N*-deacylated LPS (deON) and polysaccharide portions of LPS (PS). Gal, D-galactose; Glc, D-glucose; Hep, L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-D-*manno*-octulosonic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

3-2. Results and discussion

3-2.1 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 polysaccharide portion of LPS (PS) (Fig. 3-1) showed that 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 ethanolamine substituents (Fig. 3-3A and 3-3B) [38]. Since the LPS related compounds have no chromophores, the compounds were detected by post-column derivatization using taurine and sodium periodate [18, 32]. Unfortunately, further control of the separation energy was difficult by SAX-HPLC, because 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. 3-3C and 3-3D) using acetonitrile as the organic modifier. The separated species were also detected by the above-mentioned post column derivatization system (Fig. 3-2, 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. 3-3A and 3-3B). 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. 3-3A), was further divided into two major and two minor peaks on the RPIP-HPLC (Fig. 3-3C). The PS derivative was separated into much resolved multiple peaks on RPIP-HPLC (Fig. 3-3D), compared to the SAX-HPLC (Fig. 3-3B). 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.

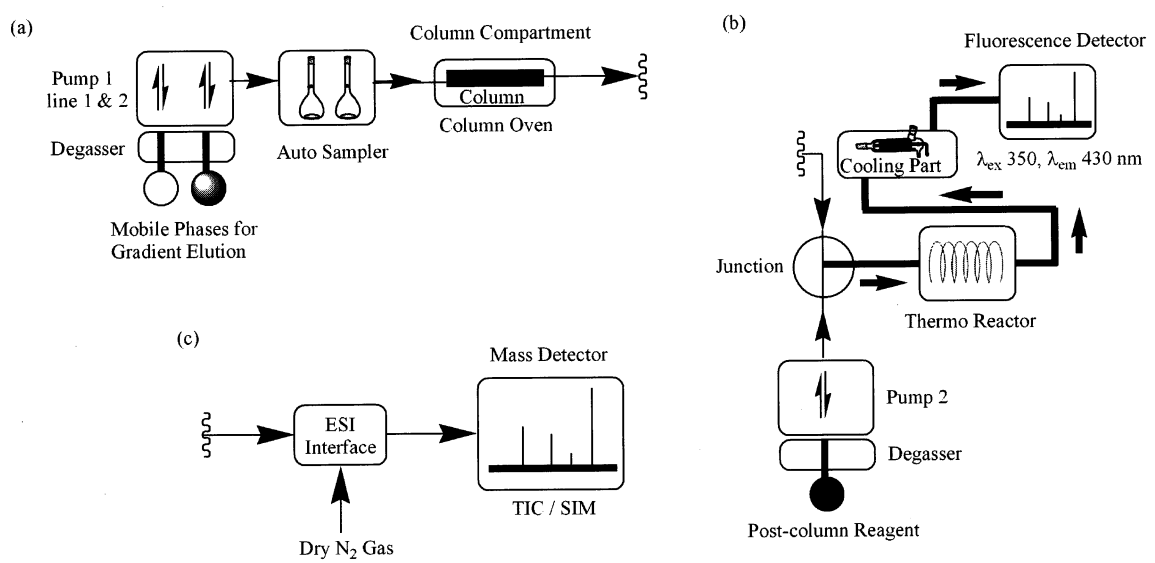


Fig. 3-2. RPIP-HPLC system with the post-column fluorescence derivatization (FLD) (from (a) to (b)) and electrospray ionization quadrupole mass spectrometry (ESI-Q-MS) (from (a) to (c)).

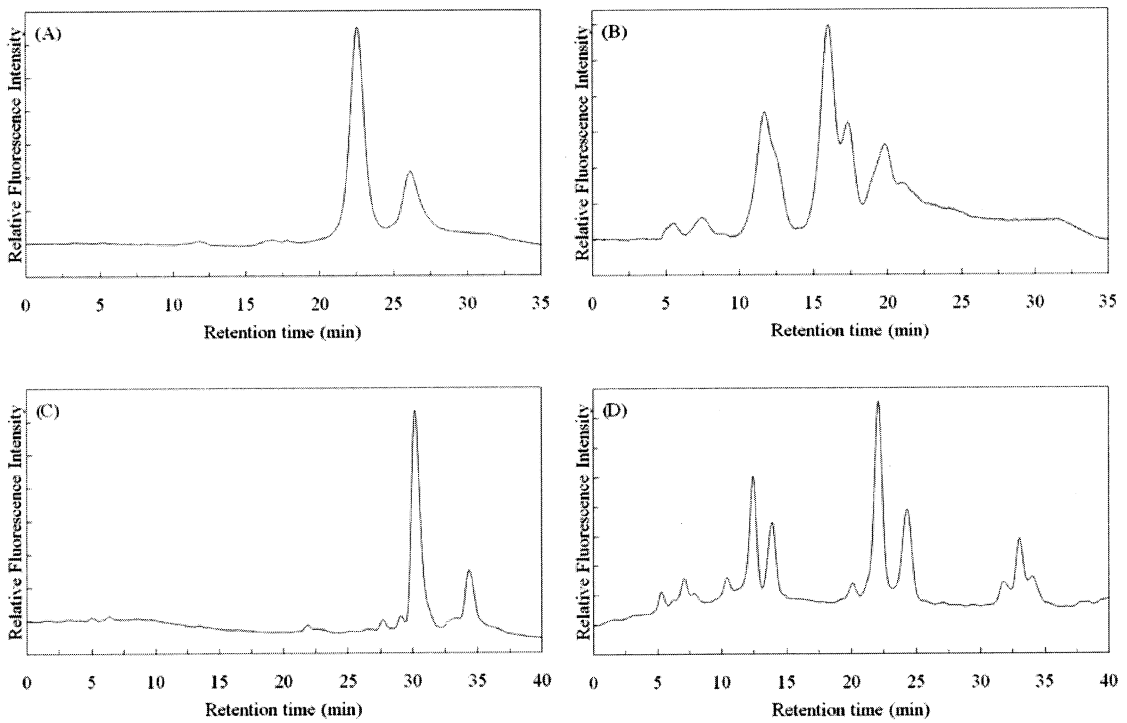


Fig. 3-3. Separation of the *O,N*-deacylated LPS (A, C) and polysaccharide portion of LPS (B, D) by strongly basic anion-exchange chromatography with post-column fluorometric derivatization (SAX-FLD) (upper) and reverse phase ion pairing chromatography with post-column fluorometric derivatization (RPIP-FLD) (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.

3-2.2 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. 3-2, from (a) to (b)). However 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.* [43] 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. 3-2, 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. 3-4A and 3-4B, 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 total chromatograms (TICs). Accordingly, selected ion monitoring (SIM) was employed to improve the detectability by using the calculated *m/z* values of individual species with different numbers of charged substituents on the polysaccharide backbones of the deON and PS derivatives (Table 3-1). The selected ion chromatogram of RPIP-HPLC/ESI-Q-MS of the deON and PS derivatives showed sharp and intense, well resolved peaks (Fig. 3-4C and 3-4D).

Table 3-1. Selected negative ions in ESI-Q-MS for deON and PS.

sample	species	proposed composition	molecular mass (Da) calculated	selected ion (*m/z*) $[M-3H]^{3-}$	selected ion (*m/z*) $[M-2H]^{2-}$	retention time (min)	rel int (%)
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	$Hex_5\ Hep_3\ KDO_2\ HexN_2\ P_3$	2186.7	727.9		-	n.d.
	deON-KDO 4P	$Hex_5\ Hep_3\ KDO_2\ HexN_2\ P_4$	2266.5	754.5		24.9	8
	deON-KDO 5P	$Hex_5\ Hep_3\ KDO_2\ HexN_2\ P_5$	2346.6	781.2		-	n.d.
PS	PS 1P	$Hex_5\ Hep_3\ KDO\ P$	1704.4		851.2	12.4	5
					851.2	14.7	5
	PS 2P	$Hex_5\ Hep_3\ KDO\ P2$	1784.4		891.2	26.9	100
	PS 3P	$Hex_5\ Hep_3\ KDO\ P3$	1864.4		931.2	30.4	39
			1864.1		931.2	32.6	8
	PS 4P	$Hex_5\ Hep_3\ KDO\ P4$	1944.4		971.2	-	n.d.
	PS+EtN 2P	$Hex_5\ Hep_3\ KDO\ P\ EtN$	1827.4		912.7	-	n.d.
	PS+EtN 3P	$Hex_5\ Hep_3\ KDO\ P_2\ EtN$	1707.4		852.7	23.1	44
	PS+EtN 4P	$Hex_5\ Hep_3\ KDO\ P_3\ EtN$	987.4		992.7	-	n.d.
	PS 2P-H_2O	$Hex_5\ Hep_3\ KDO\ P_2$-H_2O	1766.4		882.2	27.2	86
			1766.4		882.2	27.7	26
	PS 3P-H_2O	$Hex_5\ Hep_3\ KDO\ P_3$-H_2O	1846.4		922.2	30.3	28
					922.2	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	$Hex_5\ Hep_3\ KDO\ P\ EtN$ -H_2O	1809.4		903.7	-	n.d.
	PS+EtN 3P -H_2O	$Hex_5\ Hep_3\ KDO\ P_2\ EtN$ -H_2O	1889.4		943.7	23.6	44
	PS+EtN 4P -H_2O	$Hex_5\ Hep_3\ KDO\ P_3\ EtN$ -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.

In Fig. 3-3C, two minor species, 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, further resolved peaks were detected on the SIM. In the PS sample (Fig. 3-3D), PS 1P-3P and PS+EtN 3P were also detected. In the cases of PS 1P and PS 3P, further resolved 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 PS derivative [31]. In the preceding chapter, 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.

In Fig. 3-4E and 3-4F, 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 [38]. RPIP-HPLC also has 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, this method has an advantage that highly reproducible results are feasible because of the easy to operate HPLC system. Thus, the combination and selection of the above two methods can provide additional information on the diversity of non-stoichiometric substituents on LPS.

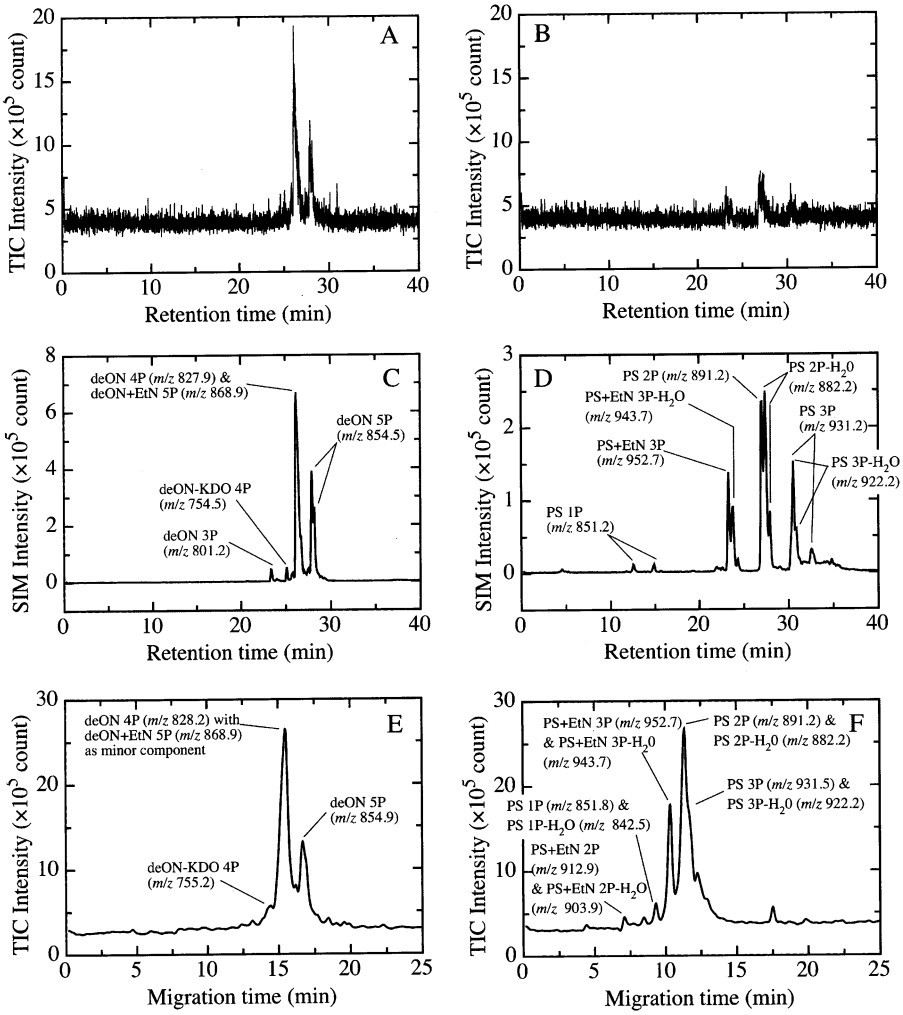


Fig. 3-4. Separation of *O,N*-deacylated LPS (A, C, E) and polysaccharide portion of LPS (B, D, F) analyzed by the reverse phase ion pairing chromatography with electrospray ionization quadrupole mass spectrometry (RPIP/ESI-Q-MS) with total ion monitoring (A, B) and with selected ion monitoring (C, D) and by the capillary zone electrophoresis with electrospray ionization ion-trap mass spectrometry (CZE/ESI-IT-MS) with total ion electropherogram (E, 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, C) and 5-20% at 0-30 min and 20-40% at 30-40 min for the polysaccharide portion of LPS (B, 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* [38].

3-3. Experimental

3-3.1. 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 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). Water was purified with a Milli-Q system from Nihon Millipore (Tokyo, Japan).

3-3.2 Samples

3-3.2.1 Lipopolysaccharide of *E. coli* C

The lipopolysaccharide (LPS) of *E. coli* C was extracted from cultured cells [29] by the phenol–chloroform–petroleum ether method [17].

3-3.2.2 Deacylated derivatives of LPS

The LPS of *E. coli* C was treated with anhydrous hydrazine (NH_2NH_2) at 37 °C for 30 min [14, 35] to form *O*-deacylated LPS (deO). This was then treated with 4 M KOH at 125 °C for 18 h [36] 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 [36, 37]. 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 [29] or flow injection analysis using an inline HPLC fluorescence detector after derivatization using taurine and sodium periodate [8], and then combined and lyophilized.

3-3.3 Methods

3-3.3.1 Strongly basic anion-exchange HPLC/post-column fluorescence derivatization (SAX-HPLC/FLD)

All anion-exchange HPLC experiments were carried out on an LC-10ADVP 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, 0 % pump 2; 27-35 min, 0 % pump, 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.

3-3.3.2 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 (line 1, 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 (line 1, 2) with degasser for the post-column reagent and a fluorescence detector was used (Fig. 3-2, 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 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. A 08.03 (Agilent Technologies, Inc., USA).

3-3.3.3 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). 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 to 2000 *m/z* for scanning mode and in accordance with Table 3-1 for selected ion monitoring mode (SIM mode). A software version was MassLynx version 4.1 (Waters, USA).

3-3.3.4 Capillary zone electrophoresis/electrospray ionization-ion-trap-mass spectrometry

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-Tap 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 were aqueous 50 % methanol and 5 μL/min, respectively.

Mass spectra were acquired using an Agilent Ion-Trap mass spectrometer (Agilent Technologies, Inc., USA), with a scanning resolution of 13000 *m/z/sec* in a standard scanning range (200-2200 *m/z*). The electrospray interface was used in negative ionization mode with the capillary voltage at 3500 V 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).

Chapter 4

Detection of submicrogram quantities of lippolysaccharides by size-exclusion chromatography/fluorescence detector with post column derivatization

Abstract

A sensitive size-exclusion chromatography (SEC) method was developed with post column derivatization using sodium periodate and taurin for the fluorescence detection of lipopolysaccharide (LPS) samples from a series of *Salmonella* mutants and several *Escherichia coli* serotypes, such as O111:B4, C, J-5, and F583. This method could detect as little as 0.1-3 μg of LPS depending on the number of component sugar having *vicinal*-diol structure. These LPS samples can be distinguished from small molecules, such as glucose, maltose, and α–cyclodextrin (α–CD). This was utilized to monitor quantitatively the total LPS content.

4.1 . Introduction

Several methods have been used to separate and analyze various LPSs, by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [44-46], agarose-gel electrophoresis [47], and gel filtration [48]. Also several methods such as silver stain [49], periodic acid-Schiff (PAS) stain [44, 50], and autoradiography [51, 52] have been used to detect the various LPS. These separation and detection methods are both a lengthy and time-consuming process for the preparation.

High performance liquid chromatography (HPLC) has been used for various polymer analysis such as carbohydrate, nucleic acid, and protein. If LPS analysis is

possible on HPLC system, it would be a generally acceptable and easy-to-use analytical method. In the past, there was a method of separating lipid A dissolved in organic solvents, such as chloroform/methanol, using a hydrophobic modified polysaccharide gel (for example, Sephadex LH-20). Moreover, it is also common to apply the aqueous gel filtration technique to the deacylated derivatives (*O,N*-deacylated LPS and polysaccharide part of LPS) which decomposed the lipid portion of LPS. However, LPS is not solved into an organic solvent but does not solve into water. Therefore, we have thought that we would be tried the chromatography method of intact LPS by using ion-paring chromatography which were found out in chapter 3. Additionally, we tried to change the behavior of intact LPS in order to determine quantity of the total LPS content. In other words, we tried to detect the intact LPS as one peak not multi-peak.

Also the detection methods of LPSs, which have no chromophore, were used periodic acid oxidation for not only PAS stain but also silver stain. This would be useful for HPLC-post column derivataization.

In this chapter, the author describes the optimal conditions of LPS separation on the siliconized diol column having the pore size of 300 Å and the post column fluorescence derivatization of the LPS. We also compare this method with SDS-PAGE and the silver-staining method.

4-2. Results and Discussion

Various LPSs were analyzed by SEC/post column fluorescence derivatization using taurine and sodium periodate. Fig. 4-1 shows a comparison of LPSs and several small molecules such as glucose, maltose, and α-CD. All LPS samples were eluted earlier than small molecules. The LPS of *E. coli* O111:B4 yielded two peaks. This phenomenon was the same result with Gel filtration using Sepharose 4B and Sephadex

G-200 columns [16]. Although LPS samples of *E. coli* F583 and J-5 have relatively short sugar chain, their retention times are very fast compared to the LPS of *E. coli* C. This phenomenon depends on their lipophilicity. In other words, their LPS would be formed bigger micelle compared to hydrophilic LPS such as *E. coli* C and O111:B4. The similar phenomena were observed for the LPS of *Salmonella* mutants (Fig. 4-2).

4-3. Experimental

A series of *Salmonella* mutant strains were supplied by Dr. Ikigai (Suzuka National College of Technology) and Dr. Haijima (National Institute of Health Sciences). The strains were cultured using a gerfermenter (10 L). LPS samples from a series of *Salmonella* mutants were extracted from cultured cells [16] by the phenol–chloroform–petroleum ether method [17]. The LPS of *E. coli* C was also extracted from the cultured cells [16] by the phenol–chloroform–petroleum ether method [17]. The other *E. coli* LPS samples were purchased from Sigma-Aldrich Japan (Tokyo, Japan).

High-performance liquid chromatography equipment was from Agilent Technologies (Agilent 1100 HPLC system: binary pump (pump 1, 2) with degasser for isocratic analysis, auto sampler with sample cooling system, column compartment used as column heater (left hand side) and cooling part of reaction mixture (right hand side), binary pump (pump 1') with degasser for reaction reagent and Fluorescence Detector). Size-exclusion chromatography separation system with the post-column fluorescence derivatization (FLD) was set as semi-micro HPLC system (Fig. 4-3).

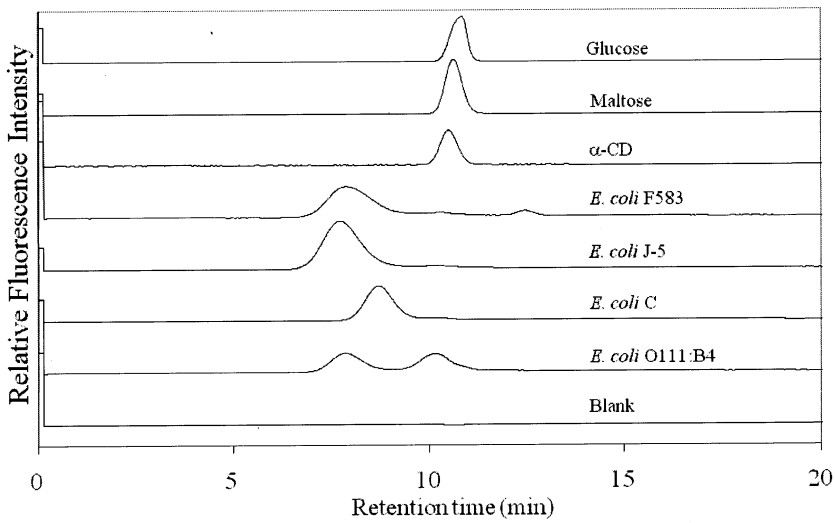
LPSs were analyzed by size-exclusion chromatography separation using a 3.0 mm ID × 250 mm stainless steel column Inertsil WP300 Diol (5 um, from GL science). The flow rate was 0.25 mL/min and the injection volume was 5 μL. Binary pump 1 was

used for delivering tributylamine (15 mM) and phosphoric acid (15 mM) adjusted to pH 7.0 with 48 % Potassium hydroxide solution. Binary pump 2 was used for delivering acetonitrite. The mobile phase was composed of the mixture of binary pump 1 and 2. The isocratic analysis was delivered at 0.25 mL/min as follows: 0 min, 20 % pump 2; 0-20 min, 20% pump 2; loop time = 20 min. Binary pump 1' was used for delivering (0.25 mL/min) the post-column reagent, which was prepared with 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 the T-junction and the mixture was introduced into the Shiseido thermo reactor (model 3019). A heating process for derivatization was performed at 100 °C in the thermo reactor. A cooling process was conducted in the same column oven with separation column. The column oven at left hand side was 30 °C for separation. The column oven at right hand side was 20°C for the cooling process. The detection was performed at an excitation wavelength of 340 nm and an emission wavelength of 430 nm. The reaction coil was 0.25 mm ID × 10 m. The cooling volume was 5 μL. Software version was Chem Station Rev. A 08.03 (Agilent Technologies, Inc., USA).

DOC-PAGE analysis was performed according to the reported procedure [53] with some modifications. The running gel consisted of 15% acrylamide, 0.4 % bis-acrylamide, and 0.9% DOC in 375 mM Tris-HCl (pH 8.8). The stacking gel was made of 3% acrylamide, 0.08% bis-acrylamide, and 0.5% DOC in 125 mM Tris-HCl (pH 6.8). LPSs were dissolved in a sample buffer containing 0.5% DOC and 20% glycerol in 80 mM Tris-HCl (pH 6.8). The electrode buffer contained 0.2% DOC, 384 mM glycine, and 50 mM Tris (pH 8.5). After pre-electrophoresis at 25 mA for 90 min, LPSs in the sample buffer were charged onto the gel and developed at 10 mA using a fresh electrode buffer. The developed gel was washed with 10% acetic acid in 40% aqueous methanol to remove DOC, and stained by the Silver Stain II Kit (Wako Pure

Chemical Industries, Osaka, Japan) after treatment with 1% periodic acid and 3% acetic acid in 40% aqueous methanol for 1.5 h at 25°C for sensitization [49].

(a)

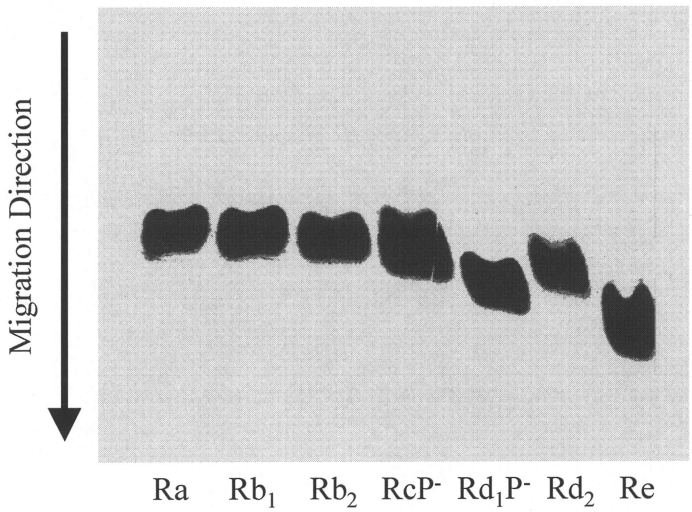


(b)

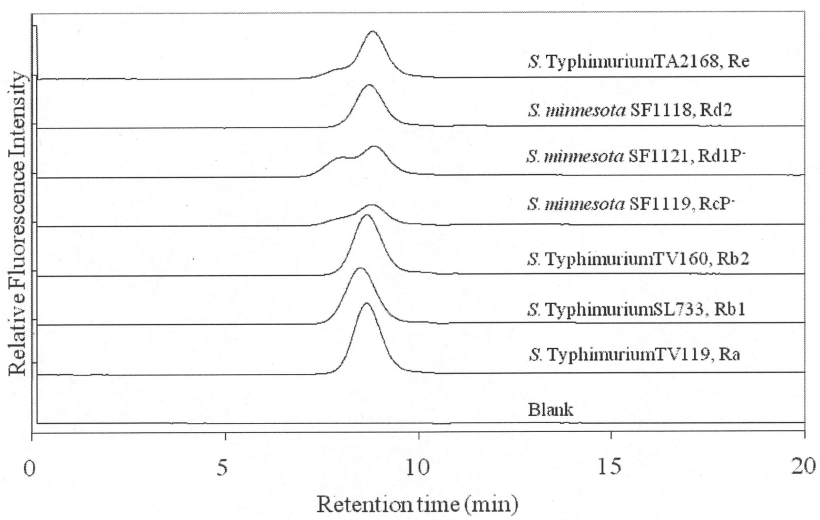
Strain (Chemotype)	O-Repeating Unit	Outer Core	Inner Core	Lipid A
E. coli F583			KDO----PPEtN α|2,4 Hep 1,5 KDO 2,6 Lipid A α α	
E. coli J-5			GlcN α|1,7 Hep KDO----PPEtN α|1,7 α|2,4 Glc 1,3 Hep 1,3 Hep 1,5 KDO 2,6 Lipid A α α α α P PPEtN	
E. coli C		Gal Glc α|1,2 β|1,3 Gal 1,2 Glc 1,3 Glc	Hep KDO----PPEtN α|1,7 α|2,4 1,3 Hep 1,3 Hep 1,5 KDO 2,6 Lipid A α α α α P PPEtN	
E. coli O111:B4	Col α|1,3 4 Glc 1,4 Gal 1,3 GlcNAc 1 α α β α|1,6 Col	Glc GlcNAc α|1,2 β|1,3 Glc 1,2 Gal 1,3 Glc	GlcN KDO α|1,7 α|2,4 Hep KDO----PPEtN α|1,7 α|2,4 1,3 Hep 1,3 Hep 1,5 KDO 2,6 Lipid A α α α α P PPEtN	n

Fig. 4-1. Analysis of several LPS and small molecules such as glucose, maltose, and α-CD. (a) SEC with post column fluorescence derivatization and (b) structures of several LPS.

a)



b)



c)

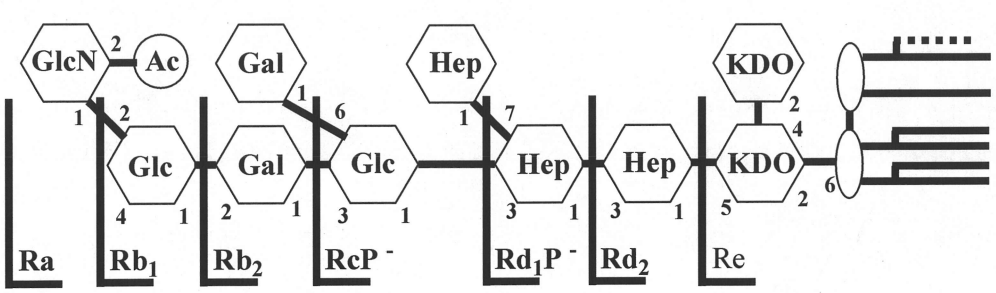


Fig. 4-2. Analysis of LPS of salmonella mutants. (a) DOC-PAGE with silver stain, (b) SEC with post column fluorescence derivatization, and (c) structures of LPS of salmonella mutants.

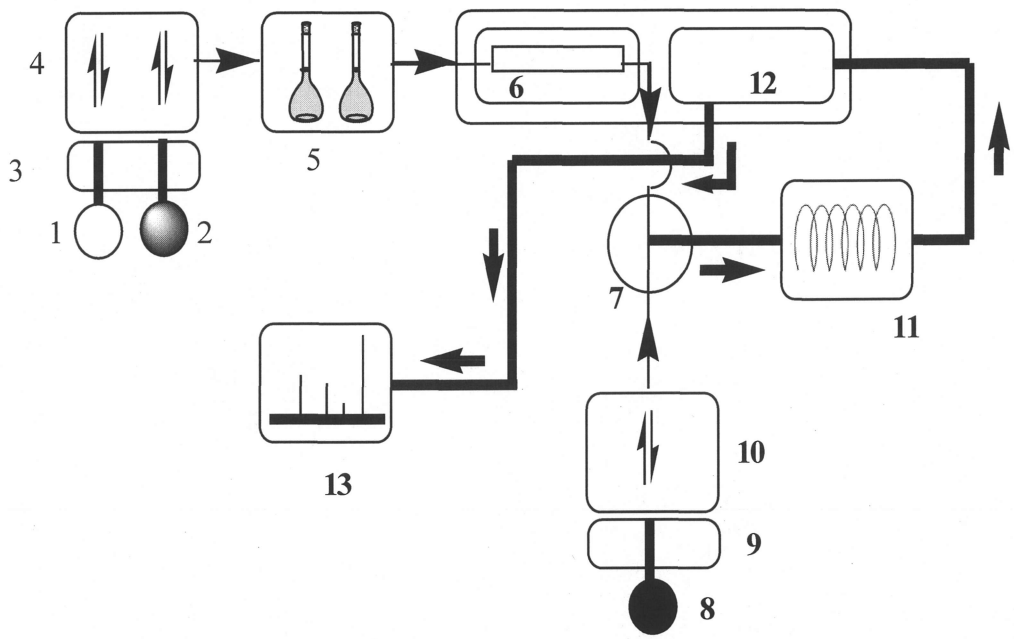


Fig. 4-3. Size-exclusion chromatography separation system with the post-column fluorescence derivatization (FLD).

Mobile phase 1, 2. Mobile phase 2, 3. Degasser, 4. Binary pump, 5. Auto sampler, 6. Column in column compartment (left hand side), 7. T-junction, 8. Post-column reagent, 9. Degasser, 10. Binary pump, 11. Thermo reactor, 12. Cooling part of reaction mixture (right hand side of column compartment), 13. Fluorescence Detector)

Summary

1. The LPS from *E. coli* C was submitted to limited chemical degradations. The derivatives of deO, deON and PS obtained were purified by gel filtration and subjected to analysis by ESI-Q-MS from a 0.1 mg/mL solution in a 50:50 mixture of methanol and 10 mM ammonium acetate (pH 6.8). The *O*-deacylated derivative showed multiple peaks of $[M-3H]^{3-}$ ions which corresponded to species having various non-stoichiometric substitution up to 8 phosphates, 2 ethanolamines, and an additional KDO on the backbone of Hex_5 Hep_3 KDO_2 $GlcN_2$ C14:0(3-OH)$_2$. The major components of the *O,N*-deacylated derivative were the species associated with 4 and 5 phosphates on Hex_5 Hep_3 KDO_2 $GlcN_2$. The polysaccharide portion of LPS also revealed to contain the species which corresponded to Hex_5 Hep_3 KDO associated with 2-4 phosphates and an ethanolamine. The present method was proved to be useful to investigate the structural diversity of enterobacterial LPS.

2. Since the diversity of non-stoichiometric substitution on the *E. coli* C LPS became clear, the analycal methods on chromatography were developed. One is the separation method by strongly basic anion-exchange chromatography (SAX) using a HiTrap Q HP column (1 mL), and the detection method has established the method of depending on post column fluorescence derivatization using 100 mM taurine and 6 mM sodium periodate (pH 7.0). The *O,N*-deacylated derivative (deON) and polysaccharide part (PS) from the lipopolysaccharide (LPS) of *Escherichia coli* C strain were separated based on the differences in the number of charged phosphate and ethanolamine substituents. The other is the capillary zone electrophoresis/mass spectrometry, and the feature can give superior separation and simultaneous characterization with a sample in small quantities.

3. Aiming at the further effective separation analysis, the reversed phase ion-paring chromatography analysis method was developed. The separation based on the difference in the number of charged phosphate and ethanolamine groups on the polysaccharide backbone was achieved by reverse phase ion-paring chromatography (RPIP-HPLC), using tributylamine as an ion-pair reagent. 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 traditional potassium phosphate buffer. The S/N ratio was low for the total ion chromatogram, however, high S/N ratio as well as good resolution were achieved 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 of RPIP-HPLC/ESI-Q-MS. The present method allows for the effective separation and inline characterization of the species corresponding to the diverse non-stoichiometric substitutions in LPS related compounds.

4. The semi-micro size-exclusion chromatography/post column fluorescence derivatization (SEC/FLD) analysis method was developed as an application for quantification of LPS. The method was effective thoroughly for a series of *Salmonella* mutants Ra-Re and several *Escherichia coli* serotypes, such as O111:B4, C, J-5, and F583. Although a limulus test is generally used as for quantification of LPS, compared with it, the place of this method, which is simple and cheap method, is the feature.

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In the present thesis, diversity analysis of LPS related compounds using ESI-MS and the following their separation analysis using high performance liquid chromatography/post-column fluorescence derivatization (HPLC/FLD), liquid chromatography/mass spectrometry (LC/MS), and capillary zone electrophoresis/mass spectrometry (CZE/MS) are investigated. The author is very happy that the present studies have made some contribution to the development of the component analysis of the general LPS related compounds and the specific interaction analysis between bacteriophage ϕX174 spike proteins and the LPS related compounds of *E. coli* C.

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List of Publications

The contents of this thesis have been published or will be published in the following original papers.

Chapter 1.

Diversity of non-stoichiometric substitutions on the lipopolysaccharide of *E. coli* C demonstrated by electrospray ionization single quadrupole mass spectrometry.

Hisaki Kojima, Minoru Inagaki, Tsuyoshi Tomita, and Teruko Watanabe.

Rapid Communications in Mass Spectrometry, **2010**, 24, 43–48

Chapter 2.

Separation and characterization of lipopolysaccharide related compounds by HPLC/post-column fluorescence derivatization (HPLC/FLD) and capillary zone electrophoresis/mass spectrometry (CZE/MS).

Hisaki Kojima, Minoru Inagaki, Tsuyoshi Tomita, Teruko Watanabe, and Satoko Uchida

Journal of Chromatography B, **2009**, 877, 1537–1542

Chapter 3.

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

Hisaki Kojima, Minoru Inagaki, Tsuyoshi Tomita, Teruko Watanabe, and Satoko Uchida

Journal of Chromatography B, **2010**, 878, 442–448

Chapter 4.

Detection of submicrogram quantities of lipopolysaccharides by size-exclusion chromatography/fluorescence detector with post column derivatization.

Hisaki Kojima, Minoru Inagaki, and Hirohito Wakashima

Analytical Biochemistry, in preparation

Other Related Publications

1) Contribution of Negatively Charged Phosphate and KDO Residues on Lipopolysaccharide to the Binding and Conformational Charge of Spike G and H Proteins of Bacteriophage ϕX174

   Minoru Inagaki, Motoko Kazusa, Tomoe Hamano, Hisaki Kojima, and Muneharu Kato

   Nova Science Publishers, Inc., *Contemporary Trends in Bacteriophage Research*, NY, USA, Horace T. Adams edition, **2009**, P. 337-351

2) Endtoxin Analysis Using HPLC/Fluorescence Detector with Post Column Derivatization.

   Hisaki Kojima and Minoru Inagaki

   Japan Kokai Tokkyo Koho **2008**-321780