Novel capillary and microchip electrophoretic methods along with mass spectrometric strategies for monitoring bacterial endotoxins

Ph.D. thesis

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„For it is here, way down at the smallest scale of matter, that little things happen that can affect our lives.”

To my Parents

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Abbreviations

APS	ammonium persulphate
ATCC	American Type Culture Collection
C12	dodecanoic acid (lauric acid)
C14	tetradecanoic acid (myristic acid)
C14-OH	3-hydroxytetradecanoic acid (3-hydroxymyristic acid)
C14unsat	unsaturated myristic acid
C16	hexadecanoic acid (palmitic acid)
C18	octadecanoic acid (stearic acid)
CAC	critical aggregation concentration
CE	capillary electrophoresis
DHB	2,5-dihydroxy benzoic acid
DOC	sodium deoxycholate
EOF	electroendosmotic flow
EU	1 endotoxin unit = 0.1 ng endotoxin
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
Gal	D-galactose
GalN	*N*-acetyl-D-galactosamine
Glu	D-glucose
GluA	glucuronic acid
GluN	*N*-acetyl-D-glucosamine
Hb	hemoglobin
Hep	heptose
Kdo	2-keto-3-deoxyoctonic acid
L-Ara4N	4-amino-4-deoxy-L-arabinose
LAL	*Limulus Amoebocyta* Lysate
LBP	lipopolysaccharide binding protein
LIF	laser induced fluorescence
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry

m/v %	mass/volume %
μ	electrophoretic mobility
NCB	nucleoid containing bacteria
PCP	phenol-chloroform-petroleum ether
psi×s	1 pound per square inch × sec = 6894.75 Pa
R type LPS	rough type lipopolysaccharide
S type LPS	smooth type lipopolysaccharide
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	*N*,*N*,*N*',*N*'-tetramethylethylenediamine
Tf	transferrin
THAP	2,4,6-trihydroxyacetophenone
Tris	tris(hydroxymethyl)aminomethane
T-unit	Tiselius-unit [10^{-5} $cm^2/V \cdot s$]
USP	United States Pharmacopeia
v/v %	volume/volume %

1. Introduction

1.1. The scope of the thesis

Endotoxins are produced by Gram-negative bacteria, which are common in the environment (particularly in water, air and dust), and moreover, naturally present in our normal intestinal flora. The free endotoxins are harmless if ingested, but may be detrimental if inhaled or introduced into the blood circulation. Endotoxemia, *i.e.*, the presence of endotoxins in the blood stream, can cause violent pathophysiological reactions. Endotoxin shock has a mortality rate of 20–50 % among hospitalized patients, and in consideration of the increasing number of resistant strains of bacteria, remaines a life-threatening syndrome worldwide. On the other hand, low doses of endotoxins (below 0.1 ng/kg body weight) are rather beneficial for the human health.

Chemically, endotoxins are lipopolysaccharide (LPS) or sometimes lipooligosaccharide (LOS) molecules, with structures varying widely from strain to strain within bacterial species. Endotoxins can, therefore, be used for serological classification of bacteria. The determination of LPS structures is essential to understand their role in bacterial virulence. This thesis focuses on the possibility of the molecular characterization of enterobacterial LPS molecules by capillary electrophoresis and microchip electrophoresis. Both techniques predominantly employ the conventional UV/VIS or fluorescence detectors. Therefore, it was a need to find suitable complexation agents, through which the primarily amphiphilic and non-UV-active LPS molecules could be solubilized and detected. Mass spectrometric studies in order to determine the molecular masses of the LPS molecular species from certain bacteria and to complete their structural characterization at the microscale level are also included.

1.2. A short historical overview of endotoxins

The history of these natural “products” of bacteria started around 1850 when it was found that infectious diseases and lethal illnesses are caused by specific living microorganisms, which can enter and multiply in the body. This modern theory of disease was formulated by Jacob Henle (see review of Beutler and Rietschel, 2003). In 1884, Robert Koch identified the causative agent of cholera, *Vibrio cholerae*. However, the

mechanism, by which the microbes created diseases, remained unexplained. Two years later, an intellectual breakthrough came from Ludwig Brieger, who discovered that microorganisms could secrete into their environment toxic products, which were sensitive to heating. He termed these products “toxins” (today broadly known as the “exotoxins” such as, for example, diphtheria toxin or tetanus toxin). However, in supernatants of living cultures of *V. cholerae* such heat-labile toxin could not be identified.

A student of Robert Koch in Berlin, Richard Pfeiffer showed that heat-killed cholera bacteria introduced to animals – as well as cholera bacteria that had apparently undergone lysis in immunised animals – would still cause lethal fever [Pfeiffer, 1892]. His experiments indicated that *V. cholerae* toxicity was not dependent on bacterial viability. These observations led him to assume that a pyrogenic (fever producing) toxin, other than protein, was released as a water insoluble part of the dissolved bacteria (see detailed review on Pfeiffers work by Rietschel and Cavaillon, 2003). He called this newly discovered heat-stable substance “endotoxin”, from the Greek *endo* meaning *within*. The advent of Hans Christian Gram’s staining method [Gram, 1884] was a further revolutionary idea, namely that bacterial species can be differentiated into two fundamentally distinct groups called Gram-positive and Gram-negative based on their cell-wall structure (Fig. 1). Through this concept we know today that endotoxins can only be synthesized by Gram-negative bacteria, including human pathogens such as *Escherichia coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus*, *etc*.

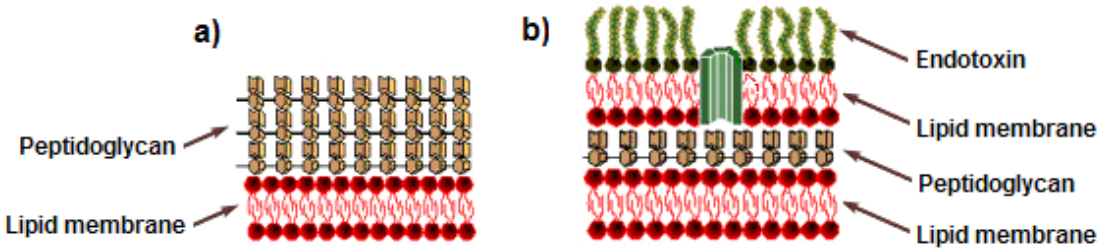

Figure 1. Schematic drawings of the cellular arrangement in a) Gram-positive bacterial cells having thick peptidoglycan layers, which are able to retain the crystal violet-iodine complex that occurs during Gram-staining, and in b) Gram-negative bacterial cells, which have a thin peptidoglycan layer, and two distinct lipid membranes; the outer lipidmembrane contains endotoxins.

After many years of effort, in 1952, Otto Lüderitz and Otto Westphal developed the method (still used today) for the extraction and purification of endotoxins [Westphal et al., 1952]. Thereafter the complete structural elucidation of endotoxins of different bacterial species became possible. The structure, biosynthesis, immunology and pathophysiology of endotoxins are still extensively studied (see reviews: Raetz and Whitfield, 2002 and Heine et al., 2001).

1.3. Chemical construction of endotoxins

Endotoxins are chemically lipopolysaccharide (LPS) or sometimes lipo-oligosaccharide (LOS) macromolecules found in the outer membrane of Gram-negative bacteria [Caroff and Karibian, 2003]. Complete (intact) endotoxins share a common basic structure (Fig. 2.) consisting of a hydrophobic lipid part termed "Lipid A", covalently attached to a hydrophilic polysaccharide part composed of a core region and a heteropolysaccharide side chain called "O-polysaccharide". The Lipid A part is integrated into the cell wall and the polysaccharide part extends outward from the bacterial cell surface (Fig. 1b). Endotoxins comprise a heterogeneous group of molecules with enormous compositional variability both, between and within bacterial strains.

Lipid A is a glycophospholipid with unique structural characteristics [Rietschel et al., 1993]. Specifically, it contains a non-reducing β-1,6-linked D-glucosamine disaccharide bis-phosphorylated at positions 1 and 4', acylated at 3,3'-hydroxyl and 2,2'-amino groups with various fatty acids, of which two (or three) are usually further acylated at their 3-hydroxyl group (see Fig. 2). All fatty acyl chains are generally saturated and their chain lengths vary between 12 and 18 carbon atoms. Treatment with alkali (*e.g.,* hydrazine) in anhydrous conditions removes the ester-linked fatty acids of Lipid A (O-deacylation) and N-deacylation can be carried out with alkaline (*e.g* potassium hydroxide) hydrolysis [Müller-Loennies et al., 2003]. The hydroxyl group at carbon 4 is free and 6' is the attachment site for the polysaccharide component. Lipid A shows high degree of similarity in various bacteria, although, fine structural variations can arise from the number, chain length and position of the fatty acids, as well as from the degree of phosphorylation and/or the presence of phosphate substituents, *e.g.*, additional sugar or ethanolamine linked to the phosphate groups. Lipid B is the term for phosphatidilethanolamine that is a membrane component often contaminating LPS preparations.

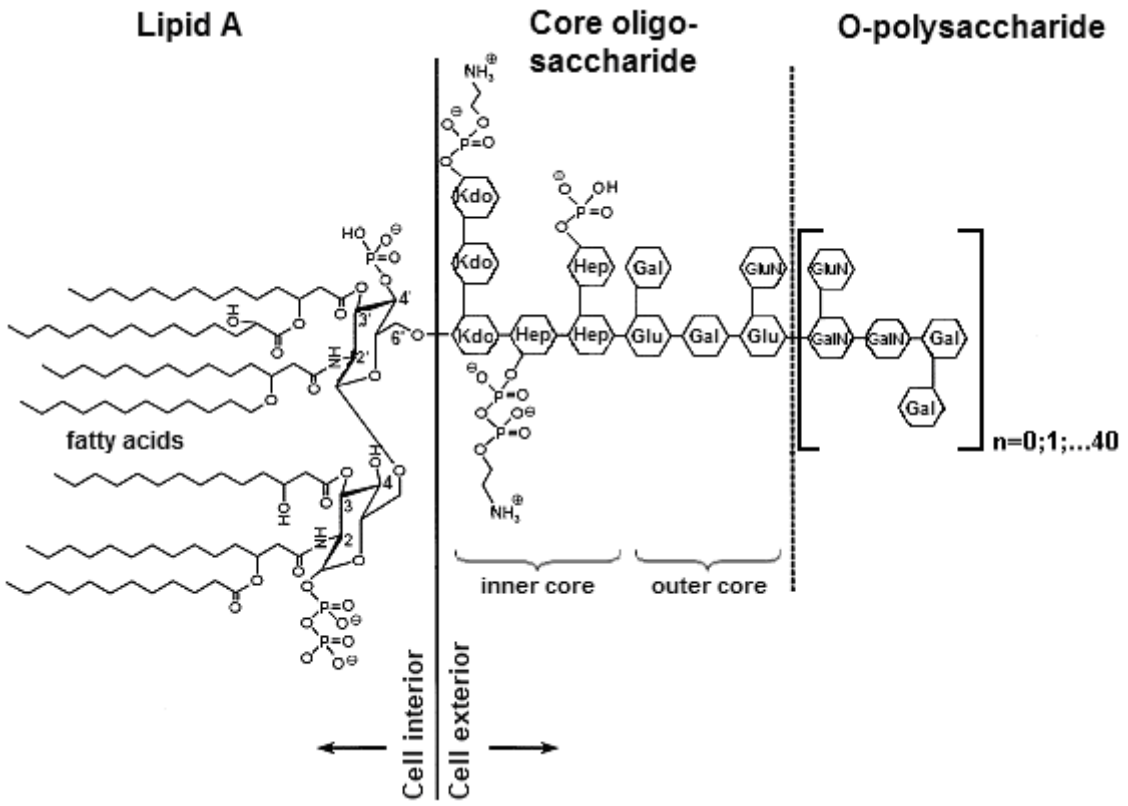

Figure 2. General structure of an intact LPS molecule represented with the endotoxin from *E. coli* O111 bacteria [Magalhães et al., 2007]. Abbreviations: Gal: galactose, Glu: glucose, Hep: heptose, GalN: N-acetyl-D-galactosamine, GluN: N-acetyl-D-glucosamine, Kdo: 2-keto-3-deoxy-octonic acid. The fatty acids are generally lauric acid (C12), myristic acid (C14), palmitic acid (C16), stearic acid (C18) and/or their hydroxyl derivatives.

The central **core** region consists of a short series of sugars and can be formally subdivided into the outer and the inner core (Fig. 2). The latter is composed of two characteristic monosaccharides: 2-keto-3-deoxy-octonic acid (Kdo) and heptose (Hep). Kdo is the connection molecule between the core and Lipid A. Inactivation of the gene which encodes the Kdo-tranferase enzyme results in bacterial lethality, thus, Kdo attachement during Lipid A biosynthesis is essential for cell growth [Belunis et al., 1995]. As a reducing terminal residue of the core Kdo has a glycosidic bond that is sensitive to mild-acid hydrolysis (at pH 3-5) [Osborn, 1963]. Heptose residues may be non-stoichiometrically substituted by a phosphate, pyrophosphate, or phosphoryl-ethanolamine group, or by another sugar to make up the inner core. The outer core generally consists of an oligosaccharide up to six sugar units, which is often branched. Common elements are D-glucose (Glu), D-galactose (Gal), N-acetyl-D-glucosamine (GluN), N-acetyl-D-

galactosamine (GalN). The complete core structure of strains of *E. coli* is generally composed of three Kdo, three Hep, three Glu and two Gal residues [Rietschel et al., 1994].

The **O-side chain** consists of repetitive oligosaccharide subunits, made up of 1–8 monosaccharide-constituents. During biosynthesis, the subunits are polymerised into blocks with length varying between 1 and 40 units and then coupled to the core [Raetz, 1990]. Deoxysugars are frequent components in O-chain structures, and the most common substituents are O- and N-acetyl, phosphate, and phosphorylethanolamine groups. The structure of repeating units (including monosaccharide diversity, possibilities of glycosidic linkage, substitution and configuration of sugars) vary widely between species and shows high species-dependency. For this reason, it forms the basis for serotype classification among the various bacterial families [Mandrell and Zollinger, 1977]. Serological cross-reactivity sometimes indicates similarities or identities between O-chain structures, also referred to as O-antigens (since they are responsible for the bacterial agglutination in O-antiserums) [Ørskov et al., 1977].

The Gram-negative bacteria can produce different chemotypes of LPS depending on the absence or presence of the O-side chains:

- The *R* or *rough*-type LPSs produced by mutant bacteria are completely devoid of an O-specific polysaccharide chain due to mutation in genes responsible for oligosaccharide biosynthesis [Rietschel et al., 1994], and sometimes even the core oligosaccharide is incomplete, as well [Heländer et al., 1988]. The *R* type bacteria create *rough* surface colonies on agar plates (Petri dish that contains a growth medium).
- The *SR* or *semirough*-type LPSs, also called lipooligosaccharides (LOSs) are produced by mutants and contain a short oligosaccharide chain with only one repeating unit.
- The *S* or *smooth*-type LPSs have various length of polysaccharide in their structure and are usually produced by the wildtype strains of members of *Enterobacteriaceae*. Their colonies cultured on agar plate have *smooth* surface.

Pure LPS preparations can be obtained from bacteria by simple procedures, typically extraction, washing and purification. For large-scale preparations the long-lasting (5-10 days) phenol-chloroform-petroleum ether (PCP) method [Galanos et al., 1969] and the phenol-water extraction procedure [Westphal et al., 1952] are applied for the extraction and purification of *R* and *S* type LPSs, respectively (see Appendix 1 for detailed

description of the procedures). Small-scale extraction of LPSs is conducted by the whole-cell lysate procedure of Hitchcock and Brown (*ca.* 40 h) leading to partially purified LPS extracted from 1-5 ml culture volumes [Hitchcock and Brown, 1983]. An LPS preparation consists of a heterogeneous mixture when isolated from a single bacterial culture [Goldman and Leive, 1980]. This microheterogeneity includes non-stoichiometric modifications of both the Lipid A and the carbohydrate moiety, depending on culture conditions of the cells during bacterial growth, or differences of polymerases and transferases playing role in O-polysaccharide biosynthesis [Tsai et al., 1983]. The intrastrain differences of LPSs in a bacterial serotype (strain) are called LPS *phenotypes*, whereas molecular species differing only in the sugars comprising the O-side chain are called LPS *glycoforms*.

The molecular mass of an endotoxin monomer is about 2-20 kDa [Li and Luo, 1998]. Endotoxins are amphiphilic molecules, thus, form aggregates (micelles) in aqueous solutions with micellar mass around 1 million Da [Aurell and Wiström, 1998]. Divalent cations such as Ca^{2+} and Mg^{2+} stabilize (via intermolecular cross-linking of phosphate groups), while detergents, like sodium dodecyl sulphate (SDS) [Oroszlán and Mora, 1963; Hitchcock, 1983] or sodium deoxycholate (DOC) [Shands and Chun, 1980] destabilize the structure. It is known that LPS aggregates can be broken down by several proteins for instance LPS binding protein (LBP), lipoproteins in plasma, albumin, lysozyme, lactoferrin, transferrin, hemoglobin, melittin and complement proteins. In vitro endotoxin binding proteins were identified by affinity- and ion exchange chromatography, immunoblotting experiments and by isoelectric focusing [Berger and Beger, 1987; Schumann et al., 1990; Schlichting et al., 1996; Li and Luo, 1997]. Generally electrostatic and hydrophobic interactions are assumed as the driving force for protein-LPS binding.

1.4. Biological definition of endotoxins

LPSs make up more than 50 % of the bacterial surface. A single *Escherichia coli* contains about 2 million LPS molecules per cell [Galloway and Raetz, 1990]. LPSs together with outer membrane proteins are essential for the stability and the function of the bacterial outer membrane, which is thus impermeable to large molecules, bile salts and hydrophobic compounds. Phosphate groups and charged sugar residues strengthen the outer membrane via crosslinking Ca^{2+} and Mg^{2+} cations.

LPSs are important virulence factors of the bacteria largely determined by the sugar sequences in the O-side chains. Rough strains are generally non-virulent. The O-antigens are immunogenic, thus easily recognized by the antibodies of the host [Rietschel et al., 1994]. However, some of the LPS phenotypes can express antigenic mimicry with human gastric epithelial cells, *i.e.*, they express host-like structures (*e.g.*, mimicry of blood group antigens, gangliosides or glycosphingolipids) [Moran et al., 2006; Vandenbroucke-Grauls and Appelmelk, 1998; Weiser and Pan, 1998]. Mimicry may have two diverging roles in pathogenesis. Autoantibodies may be induced that bind to gastric mucosa and cause damage, secondly, mimicry may cause "invisibility" of the pathogen to the host, thus hindering detection. The O-chains of smooth bacteria protect bacteria from penetration of many antibiotics.

Endotoxins remain associated with the cell wall until disintegration of the bacteria, although, small amounts of endotoxins may be released into their surroundings, especially by cultures which are actively growing during the logarithmic phase. When the bacteria lyse (*in vivo* by autolysis or external lysis mediated by the lysozyme in the complement) large amount of LPSs are set free. The growth of Gram-negative bacteria in various water storage systems (*e.g.*, infusion fluid reservoirs) is a common source of endotoxin contamination, which is usually expressed in "endotoxin units" (EU). Typically, 1 ng LPS corresponds to 10 EU [Sakai et al., 2004]. Actually, the standard laboratory autoclaving procedures have little or no effect on endotoxin levels since endotoxins are very heat stable molecules (approximately 400°C is required to break down these substances).

The presence of LPSs above 0.1 ng endotoxin/kg of body weight [Bemberis et al., 2005] in the bloodstream is highly toxic. This is called endotoxemia, which is regularly observed during a severe Gram-negative bacterial infection. The cause of "endotoxin flood" in the circulation may be the translocation of enterobacteria from the gastrointestin, notably after application of larger amounts of antibiotics or during an intestinal replacement.

Endotoxins are sensed by the so called toll-like-receptor-4 (TLR-4), which is an innate immune sensor present on macrophages especially [Poltorak et al., 1998]. The receptor activates the complement cascade, which initiates an excessive production of mediators and costimulatory molecules of inflammation such as cytokines, prostaglandins, oxygen free radicals, nitrogen oxide, tumor necrosis factor, interleukins 1 and 6 [Ulmer et al., 2002]. The resulting pathophysiological reactions are high fever, hypotension, intravascular coagulation, shock and even death by lung or kidney failure. "Endotoxic

shock" syndrome is an acute and serious disease. Because of the enormous structural variation of O-serotypes among bacteria the designing of anti-LPS antibodies for therapeutic purpose is not feasible. However, the immunisation with whole-cells of rough mutants containing the more conserved cores could be used in the creation of vaccine cross-protective for pathogenic *Enterobacteriaceae* [Lugowski et al., 1996].

Since LPSs have an immuno-stimulation activity, low amounts of endotoxin can raise non-specific resistance to infections, thus be beneficial for the host [Holst et al., 1996]. Extracts of heat-killed bacteria have been used as a vaccine in artificial fever-therapy, *e.g.*, to shrink tumors [Nauts et al., 1946].

The relationship between the molecular structure and the bioactivity of endotoxins has been studied intensively in the past. In the 1980s, the successful chemical synthesis of the lipid portion of an endotoxin molecule was an exciting advancement [Imoto et al., 1984]. Furthermore, it has been found that Lipid A is the bioactive entity of endotoxins, since *e.g.*, a chemically synthesized free Lipid A compound exhibit identical biological activity as observed with bacterial Lipid A or a whole LPS [Homma et al., 1985]. However, the toxicity properties of Lipid A can be altered dramatically by its conformation, *i.e.*, the steric factors of the fatty acids, as represented in Fig. 3. Lipid A lacking the two secondary acyl groups is non-toxic in the human system, and beside this, monophosphorylated Lipid A has 100-fold reduced endotoxin activity than has the bis-phosphorylated Lipid A compound. The „endotoxic supramolecular conformation", *i.e.*, the particular organization of Lipid A aggregates in physiological fluids has also an impact on the biological activity [Seydel et al., 2000]. These findings have importance in antagonizing endotoxin activity with LPS antagonists (*i.e.*, with compounds, which competitively prevent the binding of LPS to its receptors on target cells or which inhibit activation of cells to release bioactive mediators [Christ et al., 1999].

The fact, that endotoxins existed a billion years ago in the ancient and primitive Gram-negative bacteria, demonstrates that they are primarily produced for the survival of bacteria and are not toxic materials as such. Naturally, they are primary targets of any immune cell, since the normal function of the immune system is to prevent the body from bacterial invasions.

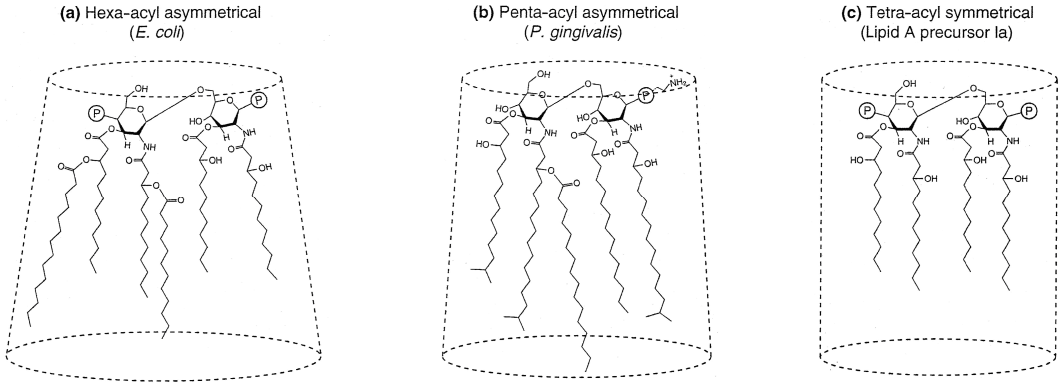

Figure 3. Correlation between molecular conformation and biological activity of Lipid A, as published by Seydel et al. in 2000. a) The hexa-acyl Lipid A with the fatty acids branched out having a conical conformation is highly active, b) the penta-acyl Lipid A having a partly conical conformation has intermediate activity and c) the tetra-acyl Lipid A precursor Ia (which is a naturally incomplete Lipid A isolated from *Salmonella*) having a cylindrical conformation is biologically inactive. P = phosphate group.

1.5. Analytical methods used in endotoxin detection and analysis

The existing methods for the detection and analysis of LPSs and LOSs can be divided into two groups, to detect bioactivity and biological effects of endotoxins, or to provide structural information about the molecules. The so called “endotoxin assays” are generally carried out by diverse in vitro and in vivo biochemical tests, *e.g.*, the USP Rabbit test and the LAL test. The structural analysis of endotoxins was problematic for a long time, since the amphipathic nature and the strong tendency of LPSs to aggregate hindered the investigation. Therefore, it was a great discovery that the Lipid A part could be cleaved from the rest of the molecule by mild acidic hydrolysis. Gel-electrophoresis is used for revealing the overall structure of intact LPS molecules, while partial structures of LPS can be thoroughly studied by *e.g.*, gas-chromatography, nuclear magnetic resonance and soft ionization mass spectrometry, which usually complement each other in resolving the entire molecule.

1.5.1 USP Rabbit test

The United States Pharmacopeia (USP) rabbit test was developed in the 1920s for

screening endotoxin levels in parenteral solutions (*e.g.*, insulin, or commercial infusion fluids). Rabbits show high endotoxin sensitivity after intravenous injection of a test solution by responding with temperature rise [Wachtel and Tsuji, 1977]. As an animal test, it has some ethical concerns, besides it has long turnaround time, it is elaborative, and expensive to perform. It should be mentioned here that rats sensitized with lead-acetate showed an increased LPS sensitivity to about 100 000 times above normal [Selye, 1966].

1.5.2. *Limulus* Amebocyte Lysate test

The *Limulus* Amebocyte Lysate (LAL) test licensed by the U.S. Food and Drug Administration (FDA) in 1973 is used to detect endotoxin contamination in biomedical products and devices, and it has replaced the Rabbit test in most laboratories. The LAL test reagent is prepared by lysing white amebocyte blood cells obtained from the American horseshoe crab *Limulus polyphemus*, an animal that coexisted with Gram-negative bacteria over million years and therefore has developed resistance to endotoxins. Its aqueous blood extract forms gel-clot with the Lipid A constituent of LPS within 15 to 45 minutes [Yin et al., 1972]. The technology can be combined with kinetic turbidimetric or chromogenic detection [Lindsay et al., 1989], which have unmatched sensitivities with limit of detection 0.03 ng/ml. However, this method in its various forms is costly and is not specific.

1.5.3. SDS-PAGE with silver staining

Slab-polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) can be used for the separation of bacterial lipopolysaccharides [Jann et al., 1975] detected with silver staining [Tsai and Frasch, 1982]. It is known that endotoxins, by nature, possess extremely high diversity of chain lengths even when isolated from a single bacterial culture (strain). This structural heterogeneity is reflected in the well-known “ladder-like” pattern on SDS-electrophoretic gels, as represented in Fig. 4. The presence of a few fast-moving bands near the front line corresponds to the simpler *R* type structures (Lipid A and core oligosaccharide), while numerous bands (both fast and slow-migrating) arranged in a ladder-pattern show *S* type structures reflecting the increasing number of repeating units present in the O-polysaccharide chains (attached to the basic Lipid A and core oligosaccharide). These individual bands are, thus, also called LPS

glycoforms. The detection of the bands is based on the reaction of aldehyde groups (formed after oxidation of the monosaccharides) and even fatty acids [Fomsgaard et al., 1990] with silver nitrate.

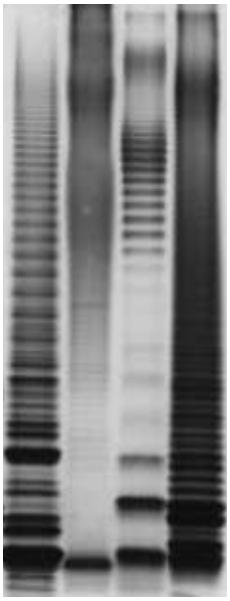

Figure 4. SDS-PAGE profiles obtained after silver staining of *S* type endotoxins from *Actinobacillus pleuropneumoniae* (lane 1), *Brucella abortus* (lane 2), *Escherichia coli* (lane 3), *Salmonella typhimurium* (lane 4) [Inzana and Apicella, 1999]. LPS size heterogeneity is reflected by variations in the numbers and intensities of the bands, which represent the number of O-chain repeating units.

The ladder-like gel pattern provides the basis for differentiation of smooth and rough endotoxins (Fig. 5). For the molecular mass determination of the endotoxin bands a comparison with a protein mass standard series can be done (Fig. 5), however, it is not at all precise because the migration properties of the proteins and lipopolysaccharides (of higher carbohydrate content) are different. For quantification of the bands, the dried silver-stained gels are scanned with a spectrophotometer which analyse and quantify peak areas.

The methodology gives good resolution and it is sensitive, since 1-2 µg of LPS can be detected, but it needs a laborious gel-preparation followed with the silver staining for the visualization of the endotoxins (see the procedure for silver staining in Appendix 2). In addition, interpretation of LPS banding patterns can sometimes be problematic due to sample overloading, and the use of protein molecular mass standards to size LPS bands is

problematic. The overall process time is *ca.* 24 hours, although, 8-10 samples can be assigned in one run.

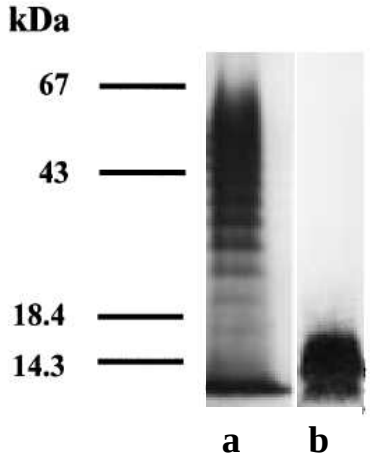

Figure 5. SDS-PAGE-separated endotoxins from a) the *S* type *E. coli* K-235, and b) from the *R* type *Bordetella pertussis* [Pupo et al., 1999]. The masses and positions of the protein bands in the Low Molecular Mass Protein Calibration kit are indicated on the left side.

1.5.4. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) spectroscopy is a first-choice method for the investigation of O-chain structures, especially when there are many repeating units. One and two-dimensional nuclear magnetic resonance of ^{1}H, ^{13}C, and ^{31}P are used to determine the sugar composition and the location of phosphate groups [Zhou et al., 2000]. In most cases, the attached core gives only weak signals, although in LOSs containing only a short O-side chain, the core signals are strong enough to interfere with those of the O-chain sugars [Larocque et al., 2003]

1.5.5. Gas chromatography

Gas chromatography combined with mass spectrometry detection (GC-MS) is used to identify monosaccharides hydrolyzed from core oligosaccharides or of O-side chains, by analysing methylated monosaccharides [Holst and Brade, 1990]. GC-MS is also applied to quantify and identify the constituent fatty acids [Binding et al., 2004]. This latter is important to distinguish, for example, two-hydroxy and three-hydroxy fatty acids, because they cannot be distinguished by a quick mass spectrometry analysis.

1.5.6. Mass spectrometry

Various soft-ionisation mass spectrometric (MS) methods, especially matrix-assisted laser desorption/ionisation (MALDI), electrospray ionization (ESI) and fast-atom bombardment (FAB) are very useful for the analysis of endotoxins [Fukuoka et al., 1997; Karunaratne et al., 1992]. Generally, the LPS substructures are separately analysed. Negative ion ESI MS is mainly used to analyse Lipid A parts, because of the presence of the easily deprotonated phosphate groups in the gas phase [Madalinski and Fournier, 2006]. MALDI-TOF MS is sensitive, but the efficiency of desorption of molecular species in a mixture depends on the choice of the matrix. MALDI MS may provide information on the length of the O-chains and on the exact number of repeating units when analysing fractions after gel-filtration of the O-polysaccharide chain [Czaja et al., 2000]. For the analysis of the fine structure of Lipid A (including phosphate content and the exact linkage of the secondary fatty acids) the LPSs are often partially deacylated, lacking either the O-linked or the N-linked fatty acids, in order to increase the solubility of LPS. MS hyphenated with online separation techniques (HPLC, CE, slab-PAGE), as well as tandem mass spectrometry, are useful tools in the structural elucidation of intact LPSs and especially Lipid A [Li and Richards, 2007; Li et al., 2005; Pupo and Hardy, 2005; Lundström et al., 2008]. However, the natural heterogeneity of most LPS preparations still complicates the interpretation of their spectra.

2. State of the art of separation techniques in endotoxin science

The determination of the structures and the structure related biological functions of bacterial lipopolysaccharides have shown much progress within the last two decades, but it is still far from complete. The fast, sensitive and quantitative characterization of endotoxins using instruments with standard optical detectors is challenging. Capillary electrophoresis (CE) and microchip electrophoresis are fast and automated methods widely used for separation and identification of biological molecules like peptides, proteins, nucleic acids, carbohydrates, viruses and cells [Hjertén, 1983; Hjertén et al., 1987; Linhardt and Toida, 2002; Kenndler and Blaas, 2001; Weiss et al., 2007], but the question arose whether these techniques can be applied also for the analysis of endotoxins. The LPSs have a net negative charge above pH 2 and thus migrate during electrophoresis. The general problem is the detection of LPS because of the lack of optically active groups or chromophores in their structures. Therefore, the *direct* detection of LPS molecules with conventional UV/VIS or fluorescence methodology is not feasible. In addition, LPSs are amphiphilic molecules and, therefore, tend to form multimeric aggregates in aqueous solutions above a critical aggregation concentration (CAC). This concentration depends particularly on the polysaccharide chain length in the LPS molecule and the ionic strength of the buffer, for example the CAC for the LPSs extracted from the *S* type *Salmonella minnesota, Salmonella typhimurium* and *E. coli O111* bacterial serotypes are 11, 14 and 22 μg LPS/ml, respectively, in a buffer solution containing 20 mM Tris and 150 mM NaCl at pH 7.5 [Aurell and Wiström, 1998]. Accordingly, suitable conditions are needed for the complete dissociation of the LPS aggregates, and appropriate reactive substances through which the LPSs can be detected.

Another essential problem faced in LPS research is the high heterogeneity of the LPS macromolecules. The direct identification of structural microheterogeneities of intact LPSs is a particular challenge, which is possible by mass spectrometric analyses only after improvements are made in the LPS sample preparation. Both microfluidic and mass spectrometric strategies – with delimitations to MALDI-TOF MS – in endotoxin analysis will be reviewed here.

2.1. The potential of capillary electrophoresis in endotoxin analysis

Electrophoresis is the migration of electrically charged particles in electric field [Tiselius, 1937; Hjertén, 1967; Vesterberg, 1989], which can be performed in slab-gel format or in microfluidic capillary formats. The commercial instrumentation of capillary electrophoresis (CE) is illustrated in Fig. 6. Typical CE systems use fused silica capillaries with 20 to 100 μm i.d., externally coated with polyimide, like those applied in gas chromatography.

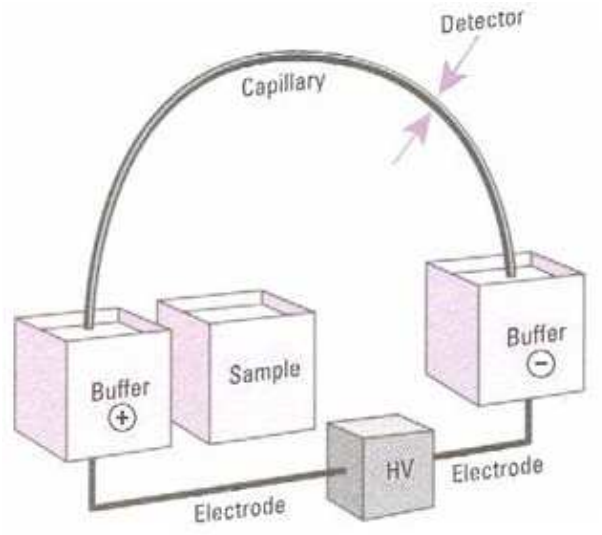

Figure 6. Schematic of the CE instrumentation. The **capillary** is hydrodynamically filled with buffer from the **buffer vial**. The sample is introduced from the **sample vial** (1-10 nL), and then the analytes start to migrate by the initiation of an electric field applied between the buffer vials by a **high voltage (HV)** power supply, which connects the two **electrodes** immersed in the buffer vials. All analytes are pulled through the capillary in the same direction (towards the **detector)** by the electoendosmotic flow (EOF) and they separate by reason of their electrophoretic mobility depending on their size and charge.

The inner surface of these capillaries are covered by silanol groups (≡Si–OH), which start to dissociate to negatively charged (≡Si–O^-) groups in contact with solutions above pH 2.5, whereas they become positively charged (≡Si–OH_2^+) below pH 2.5. The immovable surface ions attract the mobile ions of opposite charge in the buffer solution (electrolyte) by electrostatic forces, which arrange themselves into two layers called electrical double layer. The net surface charge density and the thickness of the double layer are affected by the pH and the ionic strength of the electrolyte, respectively. Upon the application of an electric field, the solvent start to migrate resulting in a plug-like

solvent flow termed electroosmotic flow (EOF). The direction and magnitude of EOF depend on the pH of the electrolyte used (*i.e.*, the charge of the surface), consequently, when the surface is negatively charged, the EOF will move towards the cathode. The ions will migrate with a resultant migration velocity owing to their own electrophoretic mobility and the mobility of the EOF, whereas neutral analytes are transported by the EOF. The mobility of EOF is given by the Smoluchowski equation [Smoluchowski, 1906]:

$$\mu_{eo} = \frac{\varepsilon_o \cdot \varepsilon_r \cdot \zeta}{\eta}$$

where μ_{eo} is the electroendosmotic mobility, ε_o is the relative permittivity of the vacuum, ε_r is the relative permittivity (dielectric constant) of the solution, ζ is the zeta potential (the electrostatic potential in the double layer) and η is the viscosity of the electrolyte. The electrophoretic mobility of an analyte at a given pH is determined by the following expression:

$$\mu_i = \frac{z_i \cdot e}{6 \cdot \pi \cdot r_i \cdot \eta}$$

where z_i is the charge of an *i* analyte, *e* is the charge of the electron, and r_i is the solvated radius of the ion. The linear velocity (v_i) of a migrating ion in an electrolyte solution is given by

$$v_i = \mu_i \cdot E$$

where *E* is the electric field strength. Upon replacing v_i with L_{eff}/t and *E* with U/L_{tot}, the mobilities of the analytes can be determined from electrophoresis experiments by the following formula:

$$\mu_{eff} = \frac{L_{eff} \cdot L_{tot}}{t \cdot U}$$

where L_{eff} is the effective capillary length to the detector, L_{tot} is the total capillary length, *t* is the migration time and *U* is the applied voltage.

If the capillary wall is pre-treated (coated) with a neutral surfactant (either a viscous polymer or a covalently attached polymer), the walls will be uncharged and the EOF will be hindered [Hjertén, 1985]. Negatively charged compounds, like proteins or endotoxins, are easier analyzed in the absence of EOF.

Despite the beneficial characteristics of CE, which include extremely high separation efficiency, very low consumption of samples and buffers, on-line detection, speed of analysis, high reproducibility and recovery when surface adsorption is minimized; the

technique has one major drawback: poor concentration sensitivity detection for the majority of biomarker analytes tested.

Up to now, only a few attempts have been made with conventional CE system (using uncoated capillaries) for the quantitative and qualitative monitoring of intact endotoxins. In one approach indirect UV detection was used applying a strongly UV-active electrophoresis buffer (Ion Phor anion PMA), detecting the non-UV-active endotoxins at 200 nm as "negative" peaks [Freitag, 1997]. The LPSs from *Salmonella minnesota* and *E. coli* bacteria were prepared in high-purity water at 1 mg/ml concentration, and detected in two peaks (for each sample) within 4 minutes, with a detection limit of 3 µg/ml. However, signals could be generated from salt anions in the sample with mobilities interfering with that of the LPSs.

Another approach was based on direct detection of LPSs from *Pseudomonas aerugunosa* at 254 nm [Freitag, 1997] and of *E. coli* O55 at 200 nm as a UV-absorbing complex with borate in the presence of SDS [Volpi, 2003]. Complex formation between the diol groups of the LPS molecules and borate anion was used to enhance the optical activity of the LPSs, and SDS to break down the LPS aggregates. The separation conditions for the separation of *E. coli* O55 LPS were 40 mM disodium hydrogen phosphate, 10 mM sodium tetraborate buffer containing 40 mM SDS, pH=9.0, capillary dimensions were 65 cm × 50 µm i.d., and the voltage was 20 kV. The electropherogram of this *S* type LPS constituted by several unresolved molecular species in a region from 18.5 to 21 min, corresponding to populations with O-specific polysaccharides of increasing mean chain lengths. Detection limit for the major component of this LPS was about 100 ng. However, this method was not tested for other LPSs.

An easily detectable marker covalently or non-covalently attached to the LPS molecules would afford a straightforward approach to enhance both solubility and detectability of LPSs. Since the Lipid A of LPSs shows the greatest similarity between various species, a labelling of this part of the molecule would be the most favourable approach to LPS detection, but, unfortunately, Lipid A is the most inert. The covalent labelling of LPSs with a fluorophore *e.g.*, fluorescein isothiocyanate (FITC) [Haas et al. 2000] is an alternative to analyze endotoxins [Freitag, 1997]. FITC is known to react readily with primary and secondary amino groups, which are also found in the core part of the LPS molecule (see in Fig. 2 in chapter 1.3). Although fluorescence detectors have high sensitivity, this technique has some inherent disadvantages, *e.g.*, the labeling procedure is time-consuming and laborious, and as reported, no significant advantages in terms of

detectability or reproducibility was obtained compared to the other methods mentioned (the detection limit was 7 µg/ml for FITC-labelled *P. aeruginosa* LPS detected as several peaks between 3 and 7 min).

2.2. The potential of microchip electrophoresis in endotoxin analysis

Since the introduction of the first microanalytic system in 1990 [Manz et al., 1990] the interest in chip based devices has increased significantly [Figeys, 2002; Dolnik and Liu, 2005]. Adaptation of CE to microchips has many advantages: a 10 to 100 fold increase in separation speed, simultaneously analyzing multiple samples, and extremely low amounts of samples and reagents needed [Lion et al., 2004]. Chip separation techniques are based on the knowledge of CE, but the microchips (fabricated from glass, quartz, silicon or plastics) contain photolitographycally defined wet-etched channels, which can be 10-40 µm deep and 50-200 µm wide [Bruin, 2000].

Bousse et al. established a miniaturized capillary gel-electrophoretic system [Bousse et al., 2001], which is now commercially available for protein separation (Bioanalyzer, Agilent Technologies; Experion, Bio-Rad Laboratories; LabChip 90, Caliper Technologies). In this system, separations are carried out in a sieving matrix which reduces the electroosmotic flow (EOF) sufficiently that the channels should not be coated. The analytes are detected by laser induced fluorescence (LIF) employing a fluorescent dyes mixed in the sieving matrix, which are able to interact with SDS. Protein–SDS complexes are, thus, labelled on-chip after entering the separation channel. Moreover, 11 samples can be analysed within 30 min.

To date, microchip electrophoresis has not been applied to the quantitative and qualitative separation of LPS.

2.3. MALDI-TOF MS in endotoxin analysis

In typical MALDI experiments, ions are generated by photon bombardment of a sample applied on a target. It is known that the mixing of a sample with an excess of a solid organic chemical, the matrix, is required for successful ionisation. This matrix-sample mixture is made to form a crystalline precipitate and is thought to absorb most of the photon energy, converting the mixture locally into vaporised material. Ions are thought to be formed in this vapour, generally by proton transfer from matrix to analyte (positive

ions) or from analyte to matrix (negative ions). Acidic compounds – like those of LPSs – typically form negative ions more efficiently. The samples are deposited on a sample targets most commonly made of stainless steel. Sample application can be performed straightforwardly by the "dried drop" method, with less than 1 µL sample volume applied. The air-dried matrix-sample preparation is inhomogeneous, and it has been shown that best ion formation occurs from particular spots.

The mass separation system in MALDI mass spectrometers is generally of the time-of-flight (TOF) type. TOF mass analyser has an indefinitely large mass range. High molecular mass compounds are run in linear mode while the low mass range of the TOF analyser provides excellent capabilities through the use of a reflectron (ion mirror) in the analyser system. The linear mode gives higher sensitivity but the resolution is more improved in the reflectron mode.

Since LPS preparations are not homogenous, LPS analyses by MALDI-TOF MS generally need improvement of sample preparation to get simpler spectra, higher intensity and increased signal-to-noise ratio. It was demonstrated that the sensitivity could be enhanced by using an ion-exchange resin (for example *Dowex*) to desalt LPS from alkali ions, and furthermore, citric acid has shown to increase solubility of the intact LPSs [Thérisod et al., 2001]. In general, the selection of matrix is empirical. According to data from literature, the saturated solution of 2,5-dihydroxy benzoic acid (DHB) matrix dissolved in citric acid has proven to be the most efficient for the ionization of endotoxins [Thérisod et al., 2001], however 2,4,6-trihydroxy-acetophenon (THAP) in methanol was also found to be useful [Sturiale et al., 2005]. Ion-source fragmentation could occur during ion flight resulting in oligosaccharide and Lipid A fragment ion signals [Sturiale et al., 2005], however, the fragment ions seen in the spectra normally result from the intrinsic heterogeneity of LPS.

3. Aims of the work

The overall aim of my study was to develop new and fast screening methods by the use of standard optical detectors for the separation and analysis of bacterial endotoxins (lipopoly- and lipooligosaccharides, LPSs and LOSs, respectively) in the field of immunochemistry or clinical vaccinology, for example antigen structure analysis. Furthermore, it was of interest to know the molecular masses and chemical structures of the intact LPSs and that of the separated LPS components. For this, our ideas and challenges were:

i) To visualize endotoxins by their protein complexes in capillary electrophoresis in order to overcome the detection and solubility problems of the amphiphilic and UV-inactive LPSs or LOSs, since it is known, that proteins can form complexes with endotoxins.

ii) To construct a qualitative and quantitative microchip electrophoretic method with high sensitivity and speed for endotoxin detection based on the facts, both, that endotoxin aggregates are dispersed by SDS in aqueous solutions, and that fluorescent dyes interact with SDS. In addition, it was of interest to investigate the capability of such microchip electrophoresis systems to be able to replace the time-consuming and laborious SDS-PAGE with silver staining.

iii) To use the new microchip technique in the characterization of partially purified endotoxins prepared directly from whole-cell lysates, *i.e.*, to develop a fast way method to detect endotoxin chemotypes. The comparison of the electrophoretic profiles with those of pure LPSs (prepared by the common procedures) was also planned to determine the correlation.

iv) To determine the molecular masses of endotoxins (from certain species of the *Enterobacteriaceae*) and to clarify their intrinsic heterogeneity by use of mass-spectrometry.

4. Materials and methods

4.1. Bacterial strains

Bacterial strains of smooth *Escherichia coli* NCB O21, O55, O83, O111, O112, 102, ATCC 25922, *Proteus morganii* O34, *Salmonella urbana* O30, *Salmonella adelaide* O35, *Salmonella minnesota* wildtype, *Shigella sonnei* phase I, *Shigella dysenteriae* 2, *Yersinia enterocolitica* O9 and of rough *Escherichia coli* NCB D31, *Salmonella minnesota* R595, *Shigella sonnei* phase II. (4303), 41, 562H and 4350 were obtained from the collection of the Institute of Medical Microbiology and Immunology, University of Pécs, Hungary.

4.2. Isolation and purification of endotoxins and Lipid A

Bacteria were grown at pH 7.2 in a 30-litre fermentor (Braun Melsungen, Germany) at 37°C for 15 h, with aeration and automatic pH correction by NaOH, in a medium containing 0.23 % beef extract, 0.2 % bacteriological peptone, 0.13 % Na_2HPO_4, 1.0 % glucose and 0.026 % $MgCl_2$. The bacterial cells were harvested by centrifugation and dried with acetone. The *S* type LPSs were extracted from the cells and purified by the hot phenol-water procedure [Westphal et al., 1952], and *R* type LPSs by the phenol-chloroform-petrol ether (PCP) method [Galanos et al., 1969], and freeze-dried at last. For detailed description of the extraction procedures see Appendix 1.

In order to obtain the Lipid A, LPSs were hydrolyzed with 0.1 M sodium acetate buffer at pH 4.4, containing 0.1 % SDS, at 100°C for 2 h. Then the solution was lyophilized, extracted once in 2 M HCl/ethanol and twice with ethanol, dried, redissolved in water, and ultracentrifuged (110000 *g*, 4°C, 1h). The sediment (containing Lipid A) was washed four times with water and lyophilized.

4.3. Preparation of partially purified endotoxins from whole-cell lysates

Partially purified endotoxins samples were prepared from whole-cell lysates according to the method of Hitchcock and Brown (1983) with modifications, as described herein.

All reagents were from Sigma (St. Louis, MO, USA), except for 2-mercaptoethanol (Bio-Rad, Richmond, CA, USA). The bacteria were grown in 1 ml Mueller-Hinton bouillon (Oxoid Ltd., UK.) at 37°C overnight. Thereafter, bacteria were collected in an Eppendorf tube and washed with 1 ml water once by centrifugation at 6000 *g* for 3 min. The pellets were resuspended in 1 ml water and then heated at 100°C for 30 min. 200 μL of this suspension was taken out in an Eppendorf tube, and lysozyme was added to a final concentration of 1 mg/ml before heating at 37°C for 30 min. During this process the peptidoglycan layers of the bacterial cell wall are disintegrated. In order to release cellular materials (like proteins, LPSs, nucleic acids, cell debris) 200 μL lysing buffer (containing 2 % SDS, 4 % 2-mercaptoethanol, 10 % glycerol, 1 M Tris-buffer, pH 6.8, and 0.05 % bromophenol blue) was added to this suspension and the lysates were incubated at 100°C for 10 min. For protein digestion, two portions of proteinase K enzyme (200-200 μg dissolved in 10 μL water) were added to the cell lysates and incubated at 65°C for 6 h (the second portion was added at the middle of the incubation time, *i.e.*, after 3 h; and the activity of the enzyme was greater than 80% of maximum). The LPS content was precipitated by adding 800 μL volume of a solution containing 3.8 mg magnesium-chloride in 50 ml ethanol, and the mixture was stored at -20°C overnight. The next day, the mixture was centrifuged at 13000×*g* for 15 min, then the sediment (containing LPS) was suspended in 30 μL deionised water and sonicated in an ultrasound bath.

4.4. Hemoglobin and transferrin

Human blood (5 ml) was delivered in a tube containing anticoagulant (EDTA) from the University Hospital in Uppsala (Sweden) and human cell-free hemoglobin (Hb) was prepared as earlier reported [Kilár et al., 1998]. Specifically, the red blood cells were separated from human blood serum by centrifugation at 3600 rpm (radius 8 cm) for 10 min. The supernatant was decanted and the pellet, containing the red blood cells, was suspended in 0.9 % NaCl solution (physiological saline) and centrifuged at 3600 rpm for 5 min. This washing procedure with NaCl solution was repeated three times. The red blood cells were then lysed by adding 800 ml of distilled water to an Eppendorf tube containing 200 ml of the suspended pellet. The hemolysate was centrifuged at 11000 rpm for 3 min to spin down cell debris and different types of organelles. The clear supernatant (containing hemoglobin at a concentration of approximately 120 mg/ml, determined with photometry) was stored in Eppendorf tubes at 4°C for a maximum of three months prior to use. The

spectra of Hb solutions were measured with a Shimadzu UV-160A spectrophotometer from 200 to 800 nm.

Transferrin (Tf), both, iron free and iron saturated, was purchased from Behring Werke (Marburg, Germany).

Other chemicals were purchased from Sigma (St. Louis, MO, USA).

4.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

1 mg purified and lyophilized LPS was dispersed in 1 ml "LPS sample buffer" (containing 0.06 M Tris-HCl at pH 6.8, 3 m/v % SDS, 10 v/v % glycerol, 5 v/v % 2-mercaptoethanol and 0.010 m/v % bromophenol blue), sonicated for 10 min and then boiled in water for 5 min. The samples were diluted 10 times with the same buffer for the analyses.

Electrophoresis was carried out in a *Laemmli* discontinuous system [Laemmli, 1970] in the Bio-Rad Mini-Protean II Dual Slab Gel system. In details, a 4 % stacking gel and a 15 % separating gel was used, both prepared from a stock solution containing 30 % acrylamide and 0.8 % N,N'-methylenebisacrylamide. The other components of the separating gel were 1.5 M Tris-HCI (pH 8.8) and 10 % SDS, and those of the stacking gel were 0.5 M Tris-HC1 (pH 6.8) and 10 % SDS. Polymerization was achieved by adding 0.005 % ammonium persulfate (APS) as initiator and 0.005 % *N*,*N*,*N*',*N*'-tetramethylethylene-diamine (TEMED) as catalizator, to each gel. 10 µl of the LPS samples were loaded on the stacking gel. The Low Molecular Mass Calibration kit (Pharmacia Biotech, Uppsala, Sweden) was run parallel to the samples. APS, TEMED and 2-mercaptoethanol were supplied by Bio-Rad (Richmond, CA, USA), all other reagents were from Sigma (St. Louis, MO, USA). The electrode buffer contained 0.124 M Tris-HC1 (pH 8.3), 0.96 M glycine, and 1.7 % SDS. Electrophoresis was performed at 40 mA (150V) until the dye front reached the bottom of the gel. Silver staining of the gels was performed by the method of Tsai and Frasch (1982), as described in detail in Appendix 2.

4.6. Capillary electrophoresis and sample preparation

A BioFocus 3000 Capillary Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA) was used for capillary electrophoresis (CE) experiments. Fused silica capillaries of 50 µm i.d. (inner diameter) were purchased from Polymicro Technologies

(Phoenix, AR, USA) and cut to the total length 28 cm (the distance from the point of injection to the outlet end) and the effective length was 23.5 cm (the distance from the point of injection to the point of detection). The capillaries were coated with non-crosslinked polyacrylamide in order to eliminate electroendosmosis and solute adsorption [Hjertén, 1985; Mohabbati et al., 2004]. See Appendix 3 for description of the coating procedure. The quality of the coating was verified by injection of a neutral marker (50 v/v % acetone in water), detecting at 260 nm, to ensure that the measured mobility corresponds to the electrophoretic mobility of the analyte with no electroosmotic component. The electrophoresis experiments were conducted in a 0.05 M Tris-HCl buffer at pH 8.5. The capillary was rinsed sequentially between successive electrophoretic runs with water for 2 min, 2 M HCl for 1 min, acetonitrile for 1 min and running buffer for 3 min. All samples were hydrodynamically injected into the capillary at 3-4 psi×s (*ca*. 2 nL) using a built-in injection system at 25°C. The applied voltage was 10-13 kV. The protein and the protein-containing complexes migrated from the cathode (negative pole) towards the anode (positive pole). Separate runs with separate injections were performed for detections at UV (205–340 nm) or at visible (340–800 nm) light, since the equipment (employing the on-column rapid spectral detector) did not allow the recording of the entire spectrum, *i.e.*, the UV and visible spectra together. The run times were around 25 min. Peak areas were calculated using the Biofocus Integrator System. The mobilities (μ) of the components was calculated in Tiselius-units (10^{-5} $cm^2/V \cdot s$) [Catsimpoolas et al., 1976] from the formula $\mu = \frac{L_{eff} \cdot L_{tot}}{t \cdot U}$, where L_{eff} is the effective capillary length, L_{tot} is the total capillary length of, t is the migration time and U is the applied voltage.

The Hb solution was diluted 100 times to a final concentration of 1.2 mg/ml (*ca*. 18 μM) with 5 mM Tris-HCl buffer, pH 8.5. The LPS solutions were prepared by dispersing 1 mg of the lyophilised LPSs in the same buffer. Mixtures of endotoxins and hemoglobin were prepared by adding lyophilised endotoxins to 1 mg/ml final concentration to 1.2 mg/ml Hb solution. Furthermore, mixtures of endotoxins and transferrin (Tf) were prepared by adding lyophilised endotoxins to 1 mg/ml final concentration to 1 mg/ml Tf solution. Protein-LPS mixtures were incubated at 37°C in a water bath to form protein-LPS complexes, and samples were run every hour by electrophoresis. Each sample was analyzed for at least 3 times.

4.7. Microchip electrophoresis and sample preparation

Microchip electrophoretic runs were performed on the commercially available Agilent 2100 Bioanalyzer system (Agilent Technologies, Waldbronn, Germany) equipped with a diode laser for fluorescence detection. In general, the Agilent Protein LabChip kits and chips developed for proteins were used in our experiments and the samples were treated roughly as it was written in the enclosed protocol, with some modifications. Briefly, to prepare LPS-SDS complex solutions, 1 mg/ml suspension of pure LPS (4 µL) or LPS samples prepared from whole-cell lysates (4 µL) were mixed with 2 µL SDS solution of 1, 2, 4 or 6 m/v % concentration. Prior to electrophoresis, LPS samples and also the "ladder" (Agilent protein standard mixture) of 6 µL were sonicated in water bath, incubated at 100°C for 5 minutes, centrifuged (6000 *g*, 15 s) and diluted with deionized water (5 times dilution for the samples and 15 times dilution for the ladder). The chip channels were filled hydrodynamically with running buffer, with help of a special syringe priming station. The running buffer contained a fluorescent dye mixed with a polydimethylacrylamide-based linear polymer as a sieving matrix. According to the enclosed descriptions, this "gel-dye mix" was prepared by mixing 650 µL gel matrix with 25 µL dye concentrate (with 630 nm as excitation and 650 nm as emission wavelengths; its chemical name was not disclosed by the vendor because of safety reasons). The composition, length, and concentration of the sieving matrix was adjusted to obtain optimal resolution within 5 to 50, 5 to 95 kDa, or 14 to 200 kDa molecular-mass range, depending on the LabChip kit used (Protein 50, 80, or 200, respectively). The high viscosity of these gels and their adsorption to the capillary wall reduced EOF to almost zero. 6 µL of each sample was loaded on the ten sample wells on the microchip, and 6 µL of the protein standard mixture was loaded on the ladder well (see chip wells in Fig. 7). The fluorescent dye present in the gel matrix had a strong complexation tendency with SDS [Bousse et al., 2001]. Unbound fluorescent dye present in the buffer filling the capillary channels was diluted at the detection, since a diluting solution (which consisted of the same linear polymer as the gel) was introduced *via* a cross-section just before the detection point, as seen Fig. 7. All samples were electrophoretically injected into the capillaries (*ca*. 40 picoliters) and substances migrated towards the anode (the positive pole found after the dilution intersection).

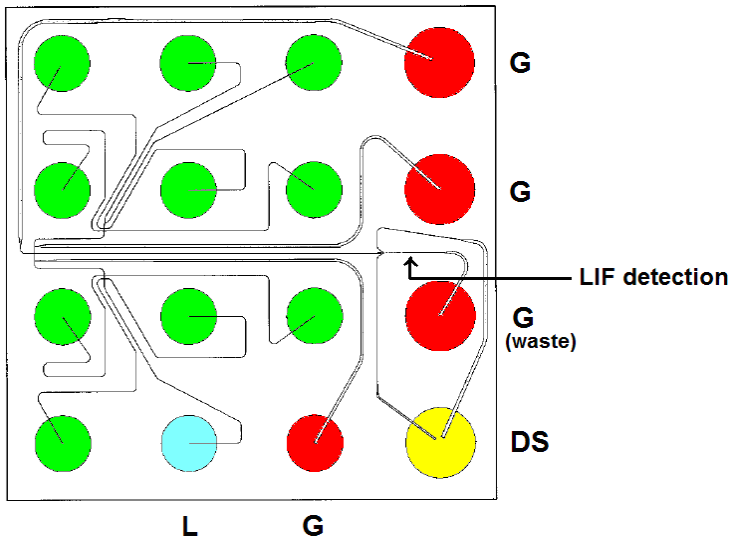

Figure 7. Chip design. The original size of the chip is 17 mm^2. The locations of the sample wells are shown in green (●). Well DS (●) is the dye dilution well and is connected to both sides of the dilution intersection. Wells G (●) are the separation buffer (gel) wells, and L (●) is the ladder (protein molecular mass marker) well. The separation channel is found in the middle of the chip, connected to the waste well, and the laser induced fluorescence (LIF) detection is found at the end of this channel. Electrodes are immersed into each well during injection and separation.

The LPS–SDS-dye complexes formed in the capillary channel were run using the 2100 Expert software. The run times were *ca*. 50 s for one sample (the collection of data started at *ca*. 10 s). Each sample was analyzed for at least 3 times in order to check reproducibility of peak numbers, peak positions, and relative peak areas. For the estimation of molecular masses of peaks appearing in the electropherograms the "ladder" (protein standard mixture) was applied of the appropriate LabChip kit. This assay used internal lower and upper markers which ensured accurate sizing of proteins (see in Fig 8). Technically, this meant that the respective marker positions in the time axis were artificially inserted in the obtained elecropherograms for data procession. The data procession by the software included baseline correction, alignment of separated runs in one chip, and estimation for molecular masses. Furthermore, the system software automatically displayed the results as gel-like images on a gray scale, to mimic a gel appearance of the peak data in the microchip electropherograms.

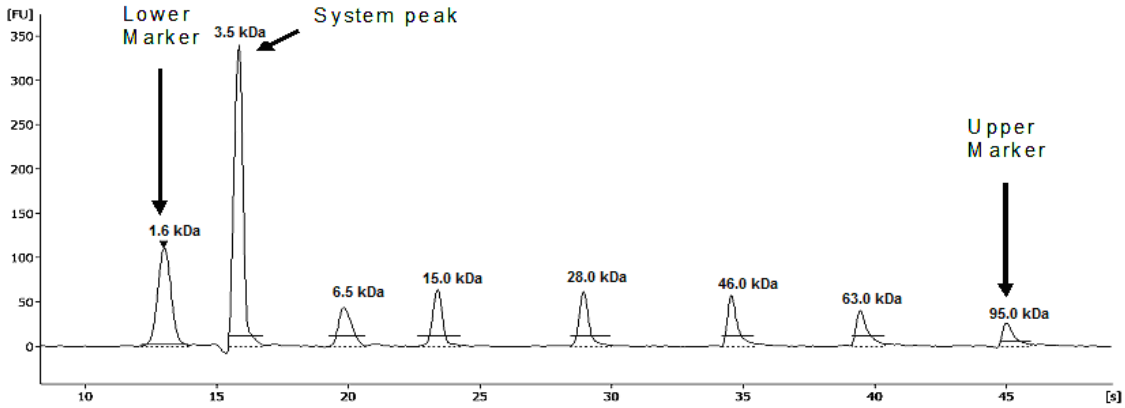

Figure 8. Microchip electropherogram of the Protein 80 Ladder (protein standard mixture), containing 7 proteins. The system peak represents the "free" SDS-dye complex in the capillary, since not all the dye molecules react with proteins. The molecular mass values are given by the software for each protein as follows: 1.6 kDa; 6.5 kDa; 15.0 kDa; 28.0 kDa; 46.0 kDa; 63.0 kDa; 95.0 kDa.

4.8. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and sample preparation

1 mg of LPS or Lipid A was suspended in 1 ml of 0.1 M citric acid solution and sonicated for 10 minutes [Thérisod et al., 2001]. 5 µL of this solution was deposited on a piece of Parafilm and desalted with some grains (*ca* 5 µL suspension) of Dowex 50WX8-200 (H^+) cation-exchange beads previously converted into its ammonium form (by boiling the beads in 0.1 M ammonium nitrate solution for 3 h). 1µL from this sample solution was deposited on a stainless steel target and mixed thoroughly with 1µL of a 10 mg/ml solution of 2,5-dihydroxy benzoic acid (DHB) matrix (dissolved in 0.1 M citric acid solution) and analyzed immediately after drying.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry **(**MALDI-TOF MS) measurements were acquired on an Autoflex II MALDI-TOF/TOF MS instrument (Bruker Daltonics, Bremen, Germany) equipped with a 1.2 m drift tube. Samples were ionized by nitrogen laser pulse (λ = 337 nm, 50 Hz). The laser power was adjusted between 20 and 45 % of its maximal intensity. Ions were accelerated through +19 kV and the spectra were recorded in the negative-ion and reflectron or linear mode over the *m/z* 1000–20000 range. Each spectrum was the sum of approximately 1000 laser shots on the same sample spot. The calibration of the instrument in reflectron or linear mode

was performed externally using a peptide calibration standard. Data processing was executed with Flex Analysis software packages (version: 2.4.). The LPS components were identified according to the molecular mass of their quasimolecular $[M–H]^-$ ions. The molecular mass measured by MS can correspond to the average mass calculated using the average atomic mass of each element of the molecule or to the monoisotopic mass calculated using the "exact mass" of the most abundant isotope for each element. The type of mass measured by MS depends largely on the resolution of the analyser. If the instrument is unable to resolve the isotopes, the mass determined corresponds to the average mass. On the other hand, if the resolution is high enough to distinguish the different peaks in the isotopic clusters, the mass determined by the instrument corresponds to the calculated monoisotopic mass [Hoffmann and Stroobant, 2007]. Evaluation of the endotoxin spectra included the summation of the masses of the sugar, phosphate and fatty acid constituents. The respective molecular masses of the constituents and the interpretation of the peaks appearing in the spectra are summarized in Appendix 4 and 5, respectively.

5. Results

5.1. Development of CE method for endotoxin detection

For the analysis of LPS in aqueous solution by standard UV/VIS detector we developed a fast and efficient capillary electrophoresis method based on the strong interaction between proteins and endotoxins [Kilár et al., 2006]. Since both, the native human hemoglobin (Hb) [Kaca et al., 1995] and the transferrin (Tf) [Berger and Beger, 1987] form complexes with LPSs, we utilized this complex formation to detect LPS in capillary electrophoresis. 1 mg/ml of LPSs was mixed with 1 mg/ml protein, incubated at 37°C for 1 h and analyzed in coated fused-silica capillaries. Electrophoretic conduction was made in Tris-HCl buffer at pH 8.5, and the electrophoretic migration of the protein-containing zones was monitored in the UV and visible region (Hb has absorption maximum at 415 nm).

The mobility of protein changed upon a complex formation with endotoxins. The electrophoretic patterns obtained after incubation with endotoxins extracted from *Escherichia coli*, *Salmonella minnesota* and *Shigella sonnei* strains were different.

5.1.1. Detection of Hb–endotoxin complexes

Figure 9a illustrates that by running the LPS from *S. minnesota* R595 alone (without any protein added to the LPS sample) no UV signal is obtained in the electropherogram. This observation was valid for other LPSs, as well (not shown). The typical separation profile of pure Hb at 205 nm and 415 nm contained one dominating peak (μ = 6.9 T-units) together with two minor peaks (see Fig. 9b). The profile obtained at 415 nm showed the same mobilities of the peaks but with lower absorbance values. The incubation of Hb with LPS extracted from *S. minnesota* R595 provided, however, peaks in two time-intervals in both the UV and the visible region, as shown in Fig. 9c. Peaks appeared at the same migration times as did the free Hb but with only one minor peak before the major peak; and an additional slightly deformed peak with a small shoulder appeared with a higher mobility (μ = 32.6 T-units). This component had similar absorption properties as free Hb, *i.e.*, it absorbed the light in both wavelength regions. Consequently, the complex of the LPS and Hb appeared as a separate peak in the electropherogram. The resolution between

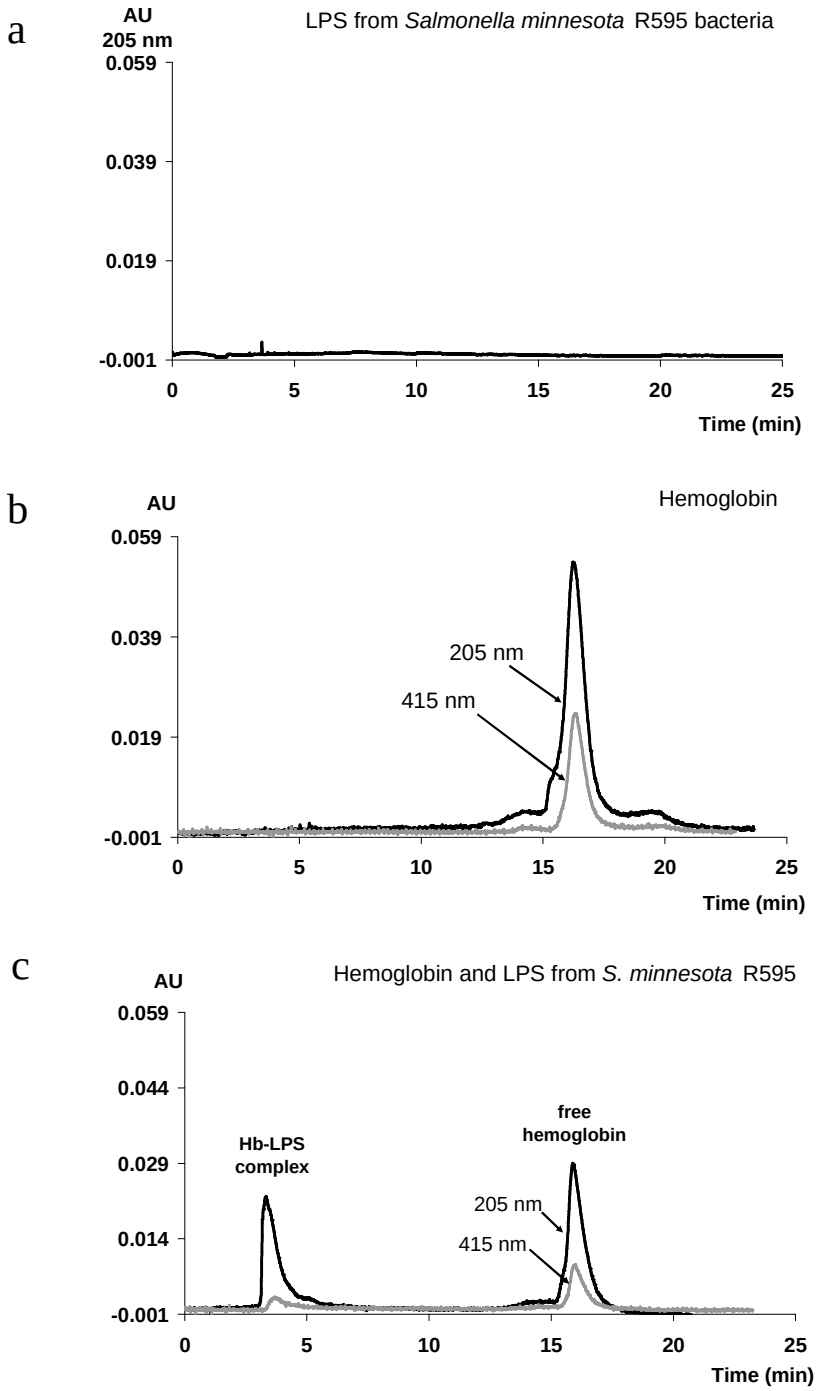

Figure 9. Capillary electrophoresis runs of a) LPS extracted from *Salmonella minnesota* R595 and b) hemoglobin (Hb) alone, and c) after mixing the two components. Sample concentrations were 1 mg/ml LPS and 1.2 mg/ml Hb. The mixture was incubated at 37°C for 1 h before electrophoresis. The fast migrating peak in c) corresponds to a complex of the two substances. Experimental conditions: capillary dimensions 23.5 cm × 50 µm, electrophoresis buffer 50 mM Tris-HCl, pH 8.5, voltage 10 kV, T = 25ºC. The hydrodynamic injections of the samples were performed with 4 psi×s. AU = absorbance unit.

the Hb-LPS complex and the pure Hb was very high, and the short migration time indicated that the negatively charged *R* type LPS molecules (with high charge to mass ratio) attached to the protein molecules caused a significant increase in the mobility of the Hb (Hb is also negatively charged at pH 8.5).Another evidence for the protein content in the complex was that the sum of the major peak areas in Fig. 9c is equal (within the experimental error, data not shown) to the major peak area of the electropherogram of Hb alone (Fig. 9b).

Upon mixing the *R* type LPS extracted from *S. sonnei* 562H with Hb, a similar complex formation was observed (see Fig. 10a). However, the major complex peak had a slightly lower mobility (μ = 30.4 T-units) compared to that of the complex in Fig. 9c. For this endotoxin a time-dependence in the complex formation was observed, since after a longer incubation (2 h) the free Hb peaks (major and minor ones, as well) disappeared and the complex peak increased (Fig. 10b). Besides, some minor components appeared after the major complex component.

Changes in the electrophoretic pattern of Hb were also observed when the *S* type endotoxin from *S. minnesota wildtype* was mixed with hemoglobin. Fig. 11 shows the electropherograms recorded in the 200–340 nm range. The Hb–LPS complex appeared as an overlapping peak between the major and minor Hb zones, with mobility of μ = 6.5 T-units. The size of this complex peak did not change when the incubation time was prolonged (not shown). The electropherogram series in the visible region showed similarly the appearance of the complex (see Fig. 11c).

Electrophoretic profiles of two *S* type LPSs extracted from *Escherichia coli* strains and one *R* type from the strain *Shigella sonnei* 41 mixed with Hb are shown in Figure 12. In the case of *E. coli ATCC* 25922 *S*-type LPS (Fig. 12a), the Hb–LPS complex peak at μ = 7.9 T-units appeared just before and partially overlapping with the free Hb peak at μ = 6.9 T-units. The broadening of the generated signal could be due to unresolved multimers of LPS populations with O-specific polysaccharides of increasing mean chain length. In the case of *E. coli* O83 strain (Fig. 12b) a better resolution of the various LPS components was visible. Several peaks (with spikes) could be seen at migration times between 2.2 and 3.6 min. Contrary to these *S* type LPSs, the *R* type *S. sonnei* 41 LPS in complex with Hb appeared mainly in two overlapping peaks (Fig. 12c) with mobilities μ = 24.5 T-units and μ = 21.1 T-units, possibly attributable to the Lipid A component and the Lipid A and core component, respectively, found in this *R* type LPS.

The LPSs had good linearity in the range of 0.05-2 mg/ml (with correlation coefficient higher than about 0.95)

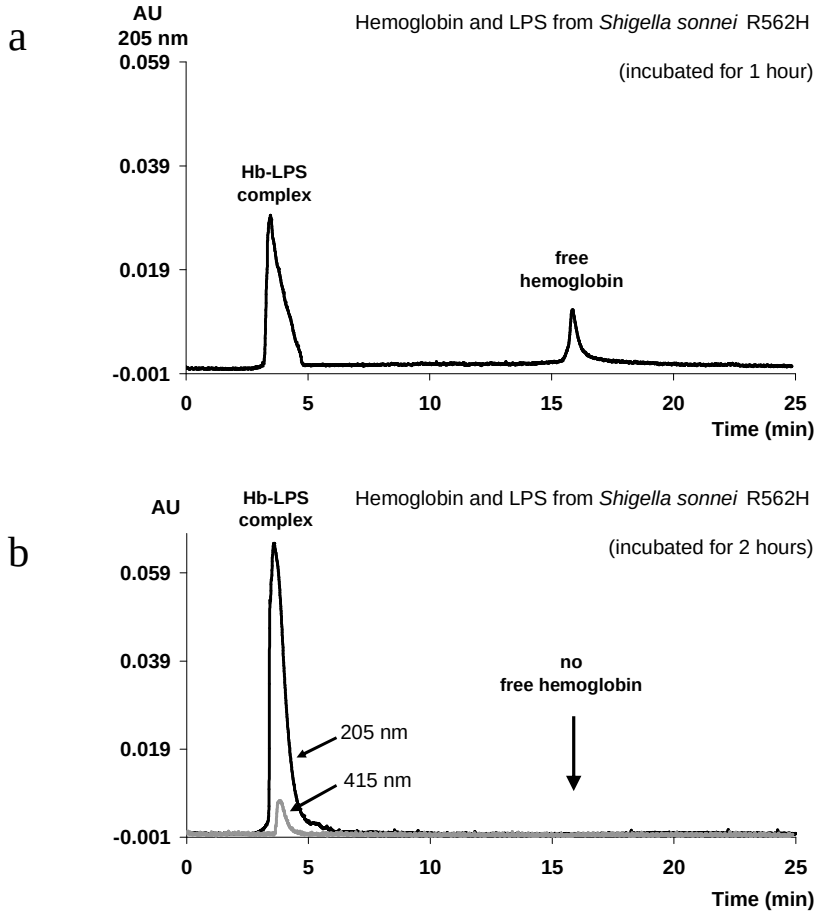

Figure 10. Capillary electrophoresis runs of a mixture of LPS extracted from *S. sonnei* 562H and hemoglobin (Hb), incubated at 37°C for a) 1 h, b) 2 h prior to electrophoresis. All Hb molecules formed complexes with LPS after two-hour incubation, since the free Hb peak disappeared from the electropherogram in b). The electropherogram after 1 h incubation was recorded only at 205 nm because of technical reasons (UV and visible spectra could not be recorded in the same run). The approximate delay between the two runs was 30–35 min. Sample concentrations and other experimental conditions were as in Fig. 9.

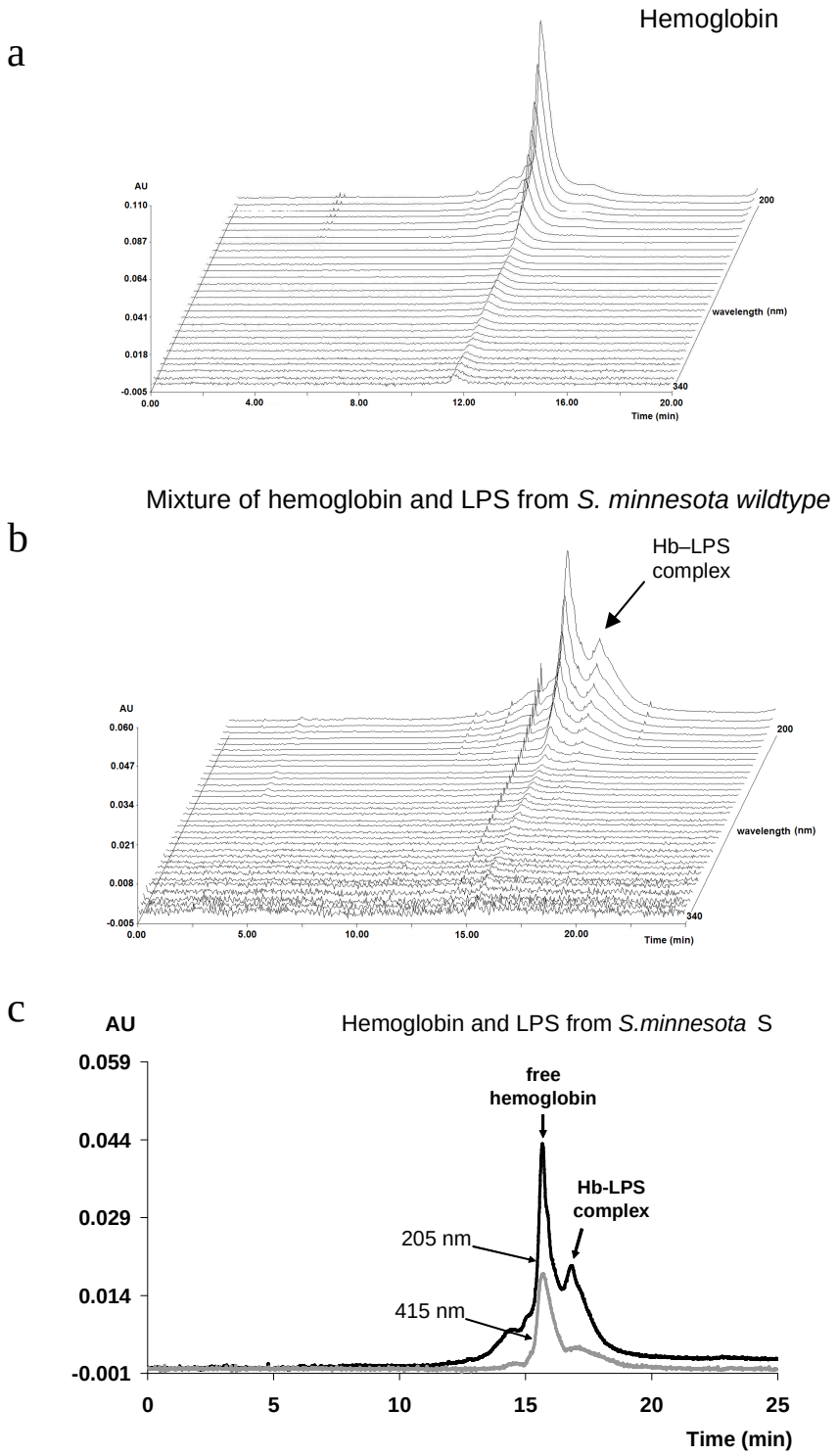

Figure 11. Capillary electrophoresis runs of a) Hb and b)-c) a mixture of Hb and LPS from *Salmonella minnesota* wildtype strain. The simultaneous electropherograms were recorded by the rapid spectral detector of the Bio-Focus 3000 apparatus between 200 – 340 nm in a) and b). The electropherogram in c) is shown at 205 and 415 nm. The mixture was incubated at 37°C for 1 h prior to electrophoresis. For other experimental conditions see Fig. 9. The Hb–LPS complex appeared as an overlapping peak after the major Hb peak.

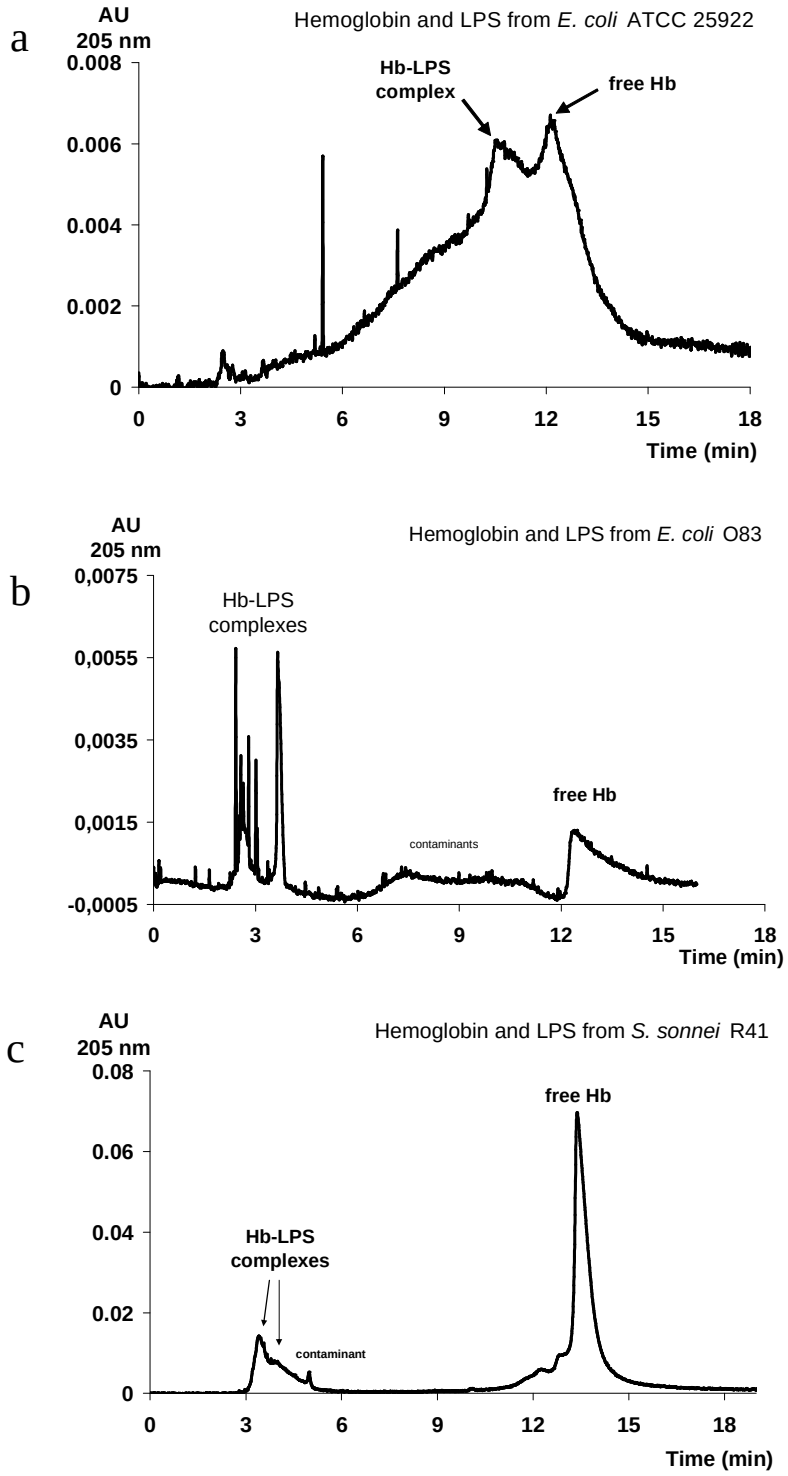

Figure 12. Capillary electrophoresis runs of Hb mixed with LPS extracted from a) *E. coli ATCC* 25922, b) *E. coli* O83 and c) *S. sonnei* 41 strains. The mixture was incubated at 37°C for 1 h before electrophoresis. Voltage 13 kV, other experimental conditions were as in Fig 9. The Hb–LPS complex peak(s) are indicated in the electropherograms, having different migration times and profiles depending on the type of LPS.

5.1.2. Spectral changes of hemoglobin

The complex formation between the Hb and LPS molecules caused a shift in the UV and visible spectral properties of Hb. Incubating Hb with endotoxin from *Shigella sonnei* 562H the absorption maxima at 275 nm and 541 nm were shifted to 269 nm and 538 nm, respectively, besides the slopes at these shifted wavelengths were depressed, as seen in Figure 13.

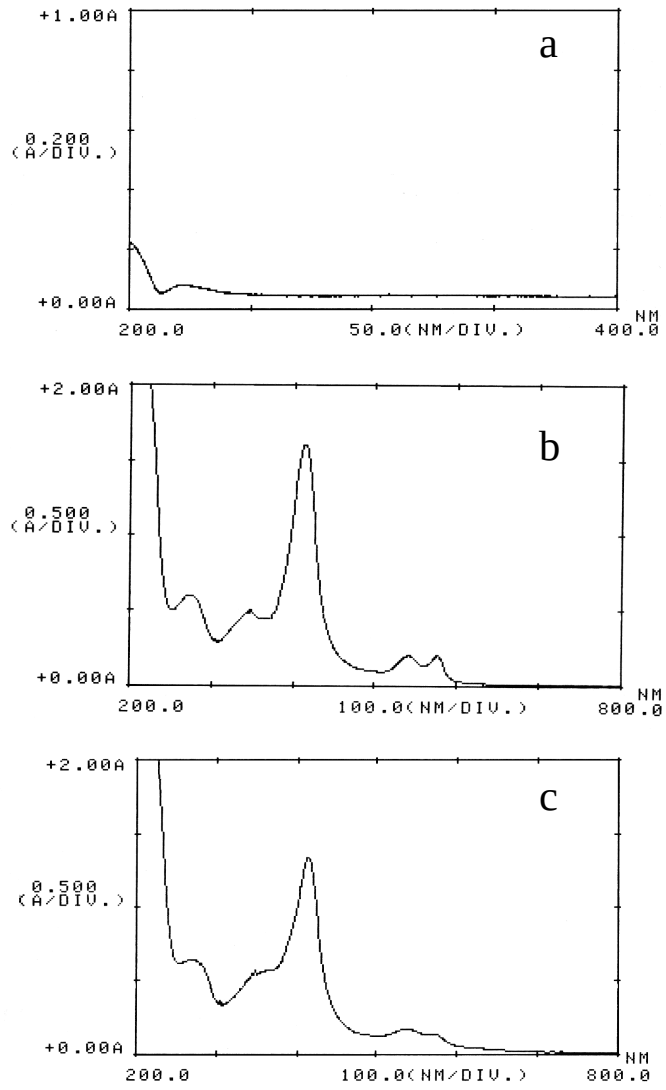

Figure 13. UV/VIS spectra and absorption values of a) 1mg/ml *Shigella sonnei* 562H LPS solution in buffer (0.05 M Tris-HCl, pH 8.5), b) 1.2 mg/ml Hb in the buffer, and c) mixture of these two components. Equipment: Shimadzu UV-160 spectrophotometer. The reference was buffer in all cases.

5.1.3. Detection of Tf–endotoxin complexes

Using the same experimental conditions, including incubation time, we also investigated the interaction of some lipopolysaccharides with iron-free or iron-saturated transferrin (Tf). The separation pattern for Tf alone, *i.e.*, in the absence of LPS is shown in Fig. 14a with peaks corresponding to the different sialoforms of Tf [Kilár and Hjertén, 1989]. Tf incubated with *S. sonnei* 562H LPS resulted in the disappearance of the Tf peaks and a simultaneous appearance of a broad peak serie between 2 and 5 min (Fig. 14b). Most probably, these peaks corresponded to the complexes of the LPS and the Tf sialoforms.

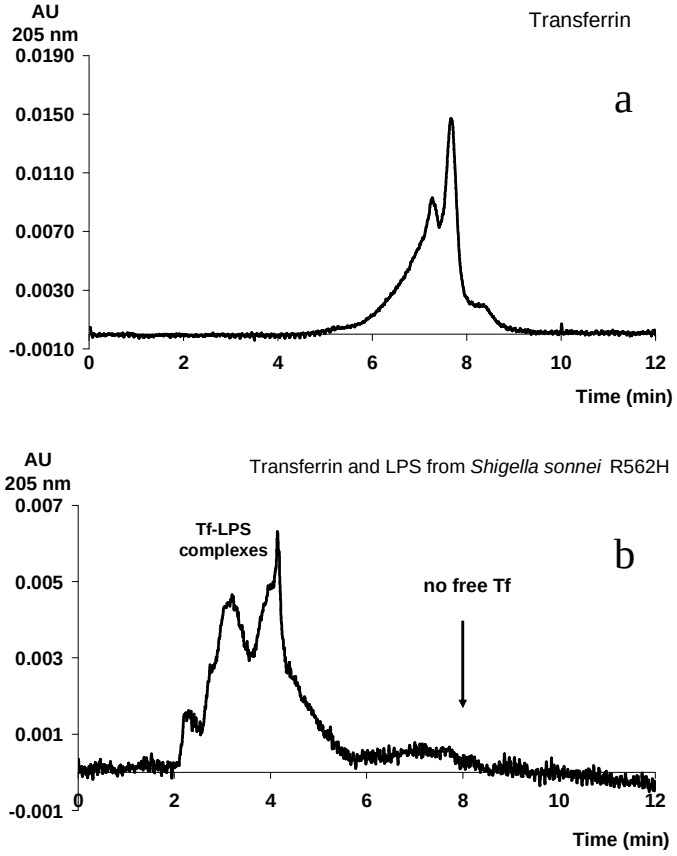

Figure 14. Capillary electrophoresis runs of a) iron-saturated transferrin (Tf) and b) its mixture with LPS extracted from *Shigella sonnei* 562H. Sample concentrations: 1 mg/ml LPS and 1 mg/ml Tf. Before electrophoresis, the mixture was incubated at 37°C for 2 h. The hydrodynamic injections of the samples were performed with 3 psi×s. Other experimental conditions were as in Fig. 9. All Tf formed complex with 562H LPS, since the free Tf peaks disappeared in the electropherogram (similarly as did the free Hb peak upon prolonged incubation with 562H LPS, see Fig. 10b).

The electropherograms for LPSs extracted from *S. minnesota* R595 and *E. coli* O83 strains incubated with Tf are shown in Fig 15a and b, respectively. The R595 LPS in Fig. 15a appeared in one small and broad Tf–LPS complex peak at about 3 min beside the free Tf peaks seen between 6 and 9 min. *E. coli* O83 LPS incubated with Tf appeared in several peaks in its complex form (spikes can also be seen), referring to smooth type LPS (Fig. 15 b).

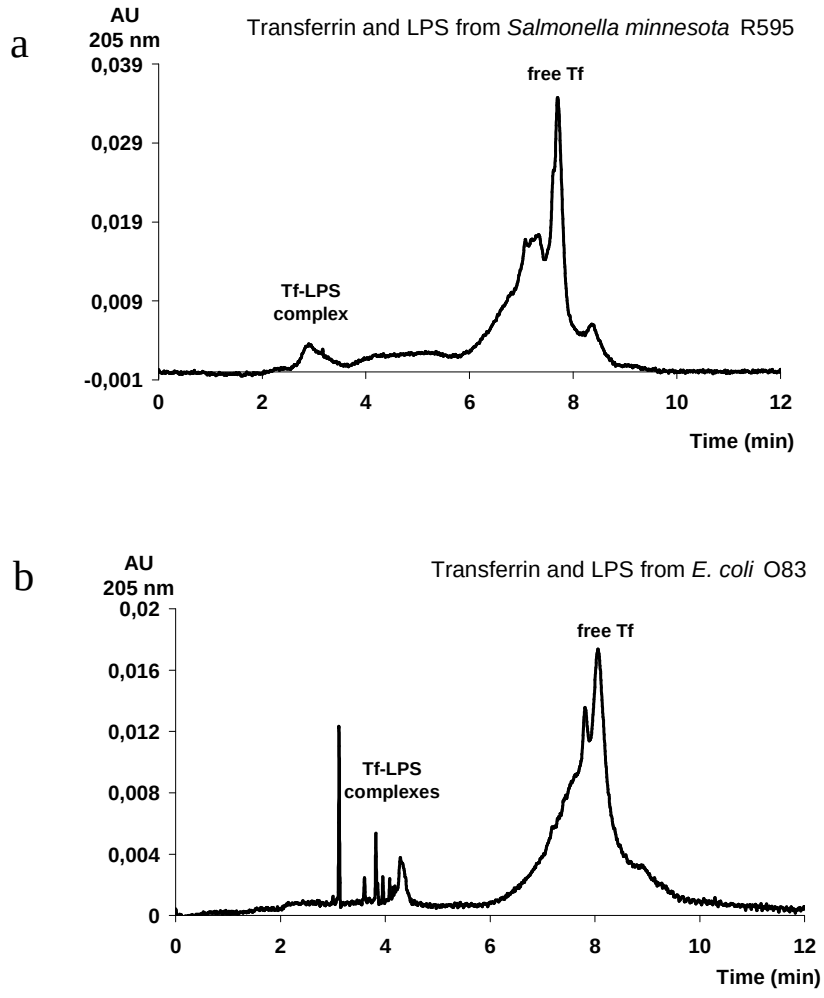

Figure 15. Capillary electrophoresis runs of a) iron-saturated Tf mixed with LPS extracted from *S. minnesota* R595 and b) iron-free Tf mixed with LPS extracted from *E. coli* O83. Sample concentrations: 1 mg/ml LPS and 1 mg/ml Tf. The mixture was incubated at 37°C for 1 h before electrophoresis. For other experimental conditions, see Fig. 14. The Tf–LPS complex peaks are indicated in the electropherograms and are comparable with the corresponding Hb–LPS complex peaks shown in Figs. 9c and 12b for *S. minnesota* R595 and *E. coli* O83 LPS, respectively.

5.2. Development of microchip electrophoretic method for endotoxin detection

For a fast and sensitive detection of endotoxins the microchip analytical technique for proteins was used to develop a new methodology. Based on the fact that LPSs interact with SDS (similarly to proteins) the detection of LPS-SDS complexes can be made using fluorescent dyes, which interact with SDS. The complexes (LPS-SDS-dye) were analysed by a microfluidic separation technique.

Microchip electrophoretic runs were performed in the commercially available Agilent 2100 Bioanalyzer system with LIF detection. Samples were injected electrokinetically in picoliter amounts. The migration of the LPS–SDS complexes was followed with the help of the fluorescent dye present in the gel (the sieving matrix), since the dye formed complex with the dodecyl-sulfate content of the LPS–SDS complexes. A Rough estimation of molecular masses of the endotoxin components by comparing the LPS peaks with the proteinaceous markers can be made in the electropherograms. The system peaks originated from the unbound fluorescent dye, although sometimes it appeared as irregular fluorescent signals with small differences in migration time, peak intensity and peak number.

5.2.1. The effect of SDS concentration on the solubilization of endotoxins

Due to the aggregation properties of the endotoxins the effect of SDS on the dispersion/solubilization of the LPSs was studied by varying the concentration of SDS initially added to the samples. Fig. 16 shows the electropherograms of the smooth-type endotoxin extracted from *E. coli* O83. The presence of large aggregates and “spikes” were seen in experiments where the LPS samples were prepared in the absence (Fig. 16a) or with low concentrations of SDS (Fig. 16b-c). The increase of the SDS concentration in the LPS sample to up to 45 mM caused sufficiently effective dispersion of the LPS aggregates (Fig. 16d). At this concentration (or above) reproducible electropherograms were obtained with peaks after the system peak corresponding to LPS microheterogeneity. Our hypothesis is that peak 1 with the largest peak area originated from an LPS molecule composed by Lipid A and core part, while the subsequent peaks contain the LPS molecular species (components) with 1, 2, …, *etc*. repeating units (peaks 2, 3, *etc.*, respectively). The “wave-like” profile composed by 20 peaks was characteristic for this *S* type endotoxin.

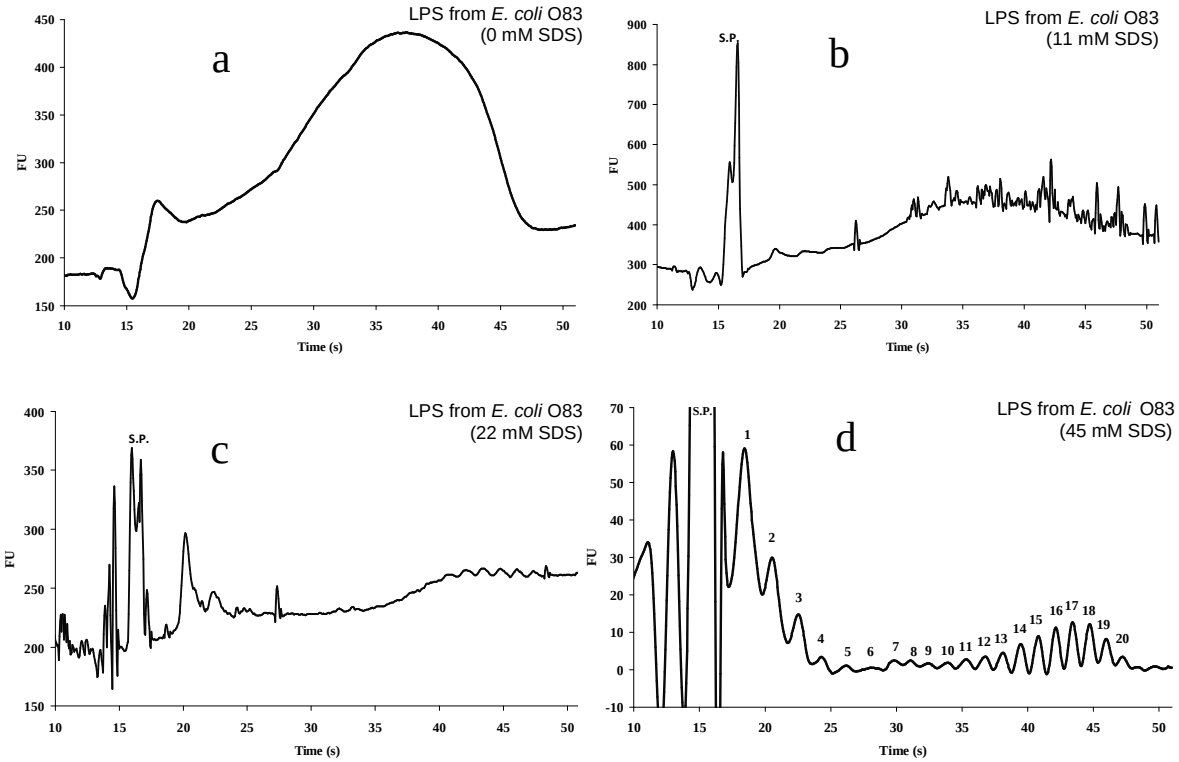

Figure 16. Microchip electropherograms of LPS from *E. coli* O83, initially solubilized in the presence of a) 0 mM, b) 11 mM, c) 22 mM and d) 45 mM SDS, respectively. Conduction was made in the Protein 80 LabChip kit. Reproducible pattern was obtained at 45 mM SDS content in the LPS sample with peaks corresponding to the endotoxin components with increasing number of O-polysaccharide repeating units. FU = fluorescence unit, S.P. = system peaks.

5.2.2. Microchip electrophoresis of pure *R* type endotoxins

In the electrophoretic patterns obtained for four rough-type endotoxins extracted from *S. minnesota* R595, *E. coli* D31, *S. sonnei* 41 and 4303, one or two components could be assigned of which the first one partially overlapped with the system peak (Fig. 17). These profiles are characteristic for R type LPSs since no “waves” appear in the electropherograms.

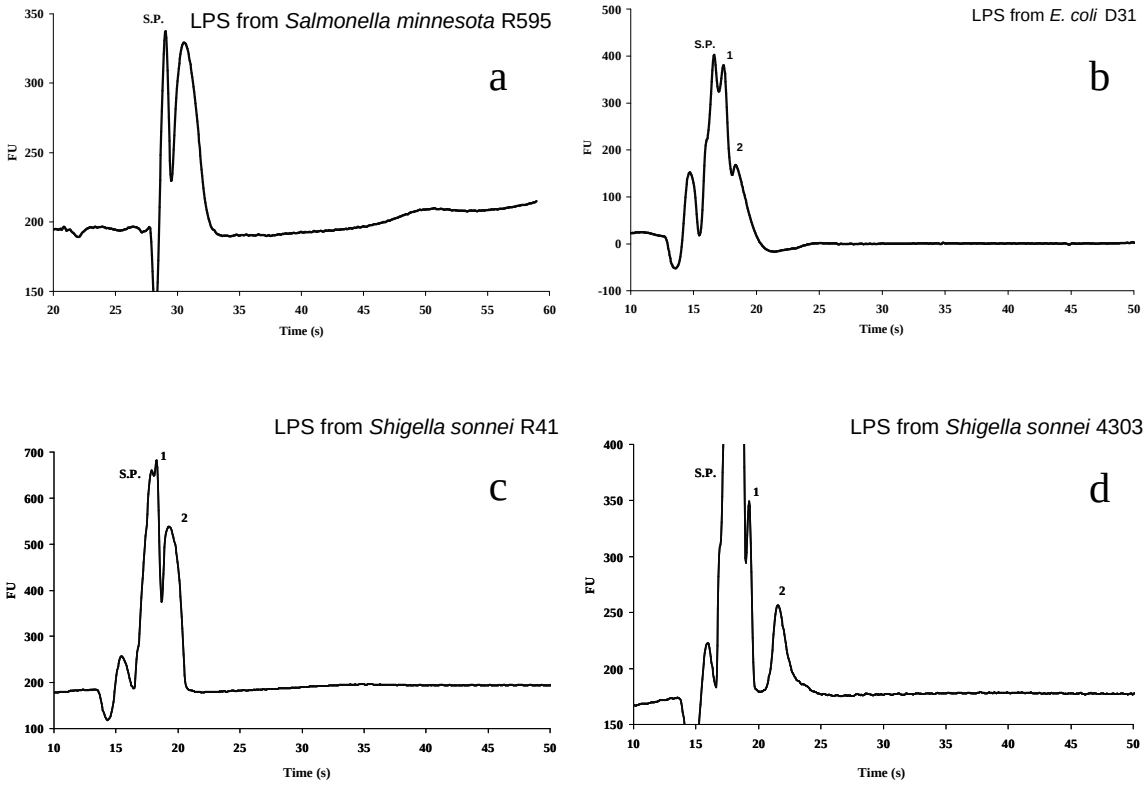

Figure 17. Microchip electropherograms of rough-type endotoxins extracted from a) *S. minnesota* R595, b) *E. coli* D31, c) *S. sonnei 41* and d) *S. sonnei* 4303. One major component was found in the case of *S. minnesota* R595, but two LPS components, partially overlapping with the system peak, were obtained in the *E. coli* D31, *S. sonnei 41* and *S. sonnei* 4303 endotoxins. The experimental conditions are the same as in Fig. 16d, except in a) where a Protein 50 Lab-Chip kit was used for the electrophoresis. S.P. = system peak.

5.2.3. Microchip electrophoresis of pure *S* type endotoxins

The microchip electrophoretic patterns of the *S* type *Salmonella adelaide* O35 LPS and *E. coli* 102 (Fig. 18) are significantly different from the pattern of *E. coli* O83 LPS. The amounts of the LPS components in case of the *E. coli* O83 first decreased then a Gaussian-like distribution appeared at the high-molecular-mass region (see Fig. 16d), while a different distribution in the "wave-like" profiles of LPS microheterogeneity appeared in case of the LPSs in Fig. 18.

The repeating unit of the *S. adelaide* O35 LPS contains colitose, glucose, galactose and N-acetyl-D-glucosamine in a molar ratio of 2:1:1:1, as described earlier [Wang and Reeves, 2000]. The chemical structure of *E. coli* 102 LPS is not yet known. At least 7

repeating units was seen for *S. adelaide* O35 (Fig. 18a), and 5 repeating units for *E. coli* 102 LPS (Fig. 18b) assuming that the first peak is composed of Lipid A and core part in both LPSs.

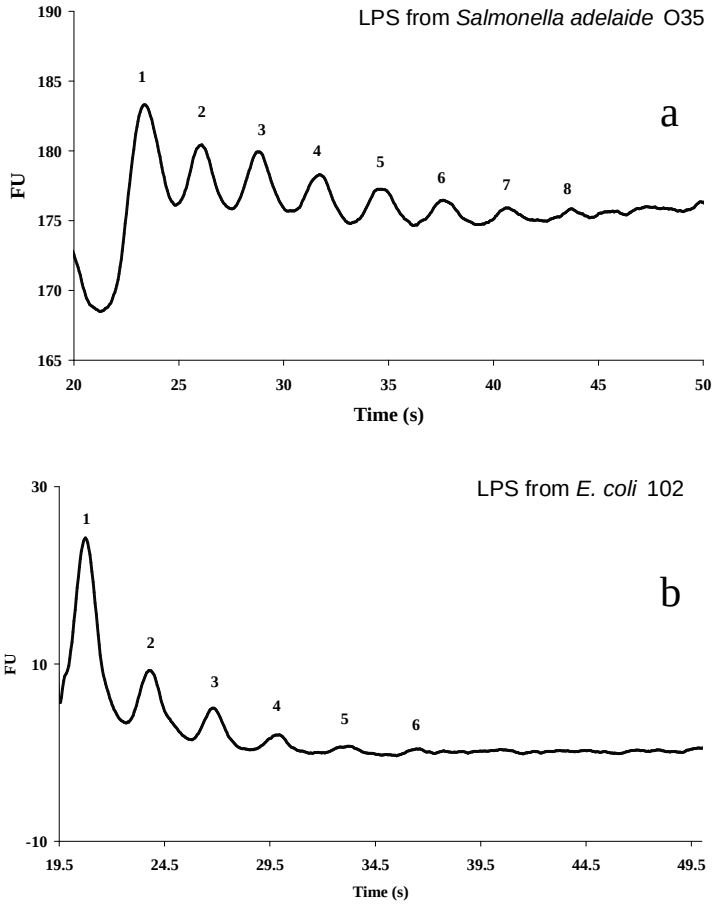

Figure 18. Microchip electropherograms of the *S* type endotoxins extracted from a) *S. adelaide* O35 and b) *E. coli* 102. The numbered peaks correspond to LPS components having oligosaccharide chains with different lengths depending on the number of the repeating units present in the LPSs. The experimental conditions are the same as in Fig. 16d.

Three different sieving matrixes, namely LabChip kits 50, 80 and 200 were compared for the separation of the *S* type *E. coli* O83 LPS. The comparative electropherograms are shown in Fig. 19. It could be seen that the resolution decreased when the kits with larger molecular mass range were applied. For further analyses of the different LPS we used the Protein 80 kit.

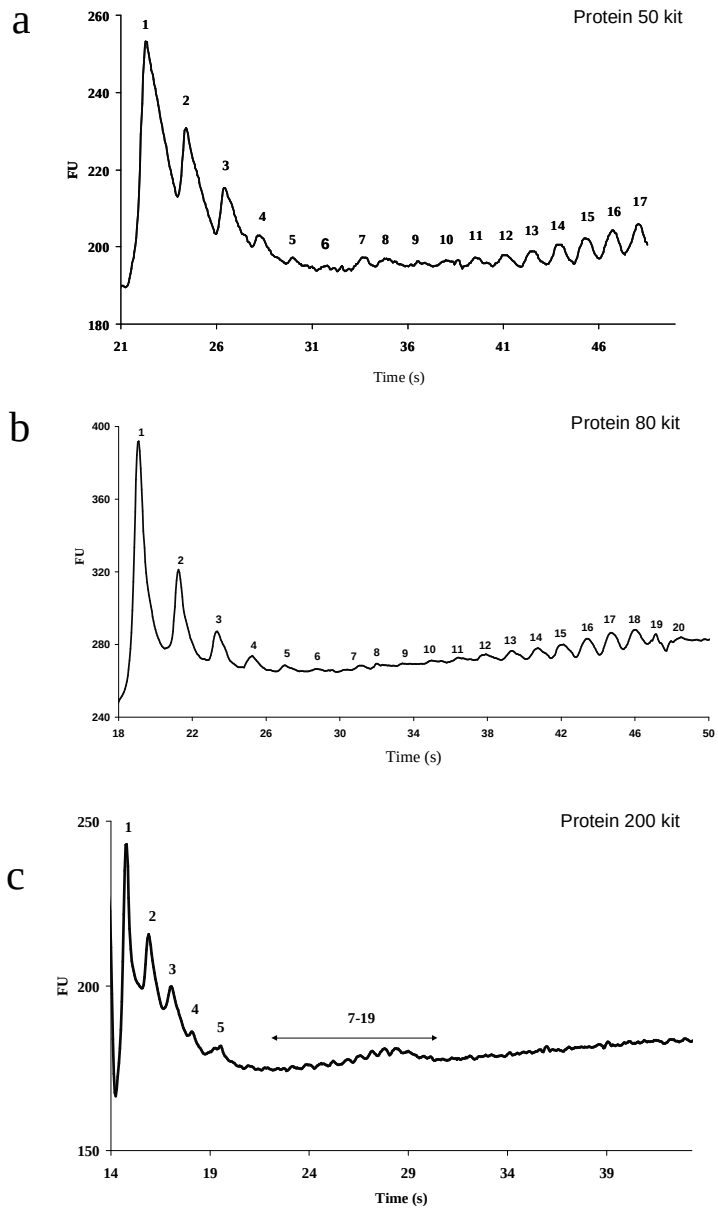

Figure 19. Microchip electropherograms of *S* type endotoxin extracted from *E. coli O83* obtained a) in the Protein 50 LabChip and b) in the Protein 80 LabChip kit and c) in the Protein 200 LabChip. Experimental conditions were the same as in Fig. 16d. The numbered peaks correspond to LPS components having oligosaccharide chains with different lengths. The LPS components appeared with different resolution in the patterns obtained with the chips with different separation ranges.

5.2.4. Comparison of SDS-PAGE and microchip patterns

The microchip software had the advantage of creating a gel-like image of an electrophoretic run. This image is very much alike to the “ladder-like” gel pattern obtained with conventional SDS-PAGE with silver staining for endotoxins. Fig. 20a and

b show the gel-electrophoretic profiles of *E. coli* O83 LPS visualized by the microchip and made by conventional slab-gel electrophoresis, respectively.

The limit of detection was determined by decreasing the amount of the endotoxins in the experiments. The major component (having the highest peak) of the LPSs were still detectable when the sample solution, applied in the chip wells, contained 30 ng LPS (at signal-to-noise ratio 3). Compared to this, the slab SDS-PAGE with silver staining needed *ca.* 2 µg LPS applied in the gel well for proper detection of the bands.

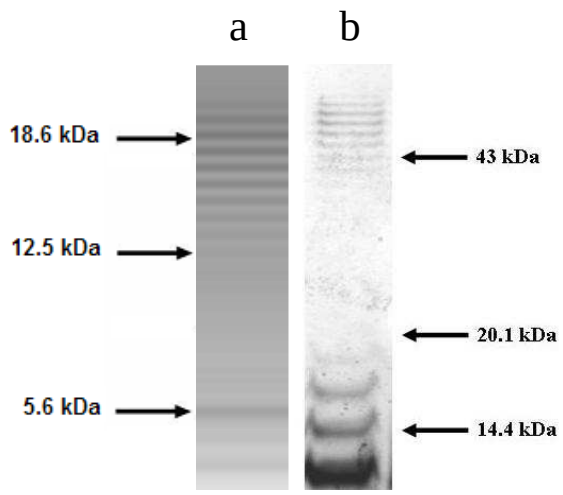

Figure 20. SDS-PAGE patterns of LPS from *E. coli* O83. The first pattern (a) is a gel-like transformation of the peaks appearing in the microchip electrophoregram (see Fig. 16d), the second (b) was obtained with conventional SDS-PAGE after silver staining. The arrows indicate the positions of protein molecular mass standards run in the respective methods. The two patterns are similar.

5.2.5. Molecular mass estimation of endotoxins

The determination/estimation of the molecular mass values characterizing the LPS components was made in three ways. Knowing the composition of the LPSs, the molecular masses of the components (with increasing numbers of repeating units) can be calculated. The data processing software of the Bioanalyzer (see the standard protein molecular mass "ladder" in Materials and Methods) had provided "molecular mass" values for each LPS components that would basically characterize proteins with the same migration properties. Similarly, using the protein standards' positions in the SDS-PAGE, the molecular masses can be estimated from the gel-pattern (see *e.g.*, the arrows on the

right side in Fig. 20). The calculated and "estimated molecular masses", as well as the relative peak areas (obtained by the microchip analysis) for each component of *E. coli* O83 LPS are given in Table 1. The relative peak areas were deduced by manual integration, where the largest peak area was considered to be 1000 arbitrary units. The relative standard deviation for the molecular masses (calculated from the migration times) characterizing the LPS components was less than 1.6 %.

Table 1. Molecular mass data of components (peaks) appeared in the electrophoretic pattern of the LPS extracted from *E. coli* O83 bacteria.

		SDS-PAGE	SDS-microchip	
Peak number	**Theoretical molecular mass* (kDa)**	**Estimated molecular mass** (kDa)**	**Estimated molecular mass*** (kDa)**	**Relative peak area**
1	3.9		6.1	1000
2	4.7		10.0	243
3	5.6	14.4	14.5	185
4	6.5		17.9	50
5	7.3		22.4	54
6	8.2	20.1	25.9	12
7	9.1		32.1	23
8	9.9		35.7	23
9	10.8		39.5	12
10	11.7		45.0	15
11	12.5		49.0	42
12	13.4		54.5	66
13	14.3		59.1	89
14	15.1	43	64.2	131
15	16.0		71.9	185
16	16.7		79.6	232
17	17.7		87.3	212
18	18.6		95.0	162
19	19.5		100.8	97
20	20.3		107.1	42

* Calculated molecular masses according to the chemical structures found in the literature [Rietschel et al., 1994; Jann et al., 1994] along with our mass spectrometric measurements (see chapter 5.4), supposing that peak 1 with the largest peak area represents the component composed by the Lipid A and the core part, and the subsequent peaks refer to components differing with one repeating unit in their polysaccharide side chains.

** Molecular masses estimated from the positions of the protein molecular mass standard (LMW Electrophoresis Calibration Kit, Pharmacia Biotech).

*** Molecular masses estimated from the positions of the protein molecular mass standard (Agilent Protein 80 Ladder).

It can be seen from Table 1 that the calculated molecular masses for each LPS components are lower than the estimated molecular masses from either the SDS-PAGE, or the microchip runs. The relative peak areas of components in the microchip electropherogram were proportional with the intensities of the bands in the gel-pattern of the SDS-PAGE (judged visually), however, quantification could only be made for the microchip runs by the microchip software.

5.3. Microchip electrophoresis of partially purified endotoxins prepared from whole-cell lysates

The microchip electrophoretic method developed by us for pure LPSs was also tested for partially purified endotoxin samples prepared from whole-cell lysates. For this, bacterial strains were cultivated in 1 ml medium, lysed and subjected to proteolytic digestion. The obtained protein-free LPS samples were directly analysed (without further purification) by microchip electrophoresis using the Agilent Protein 80 LabChip kit.

5.3.1. Microchip electrophoresis of *S* type endotoxins prepared form whole-cell lysates

Fig. 21 shows the electrophoretic profile of the partially purified LPS in *E. coli* O83 whole-cell lysate in relation to the profile of the same, but purified LPS.

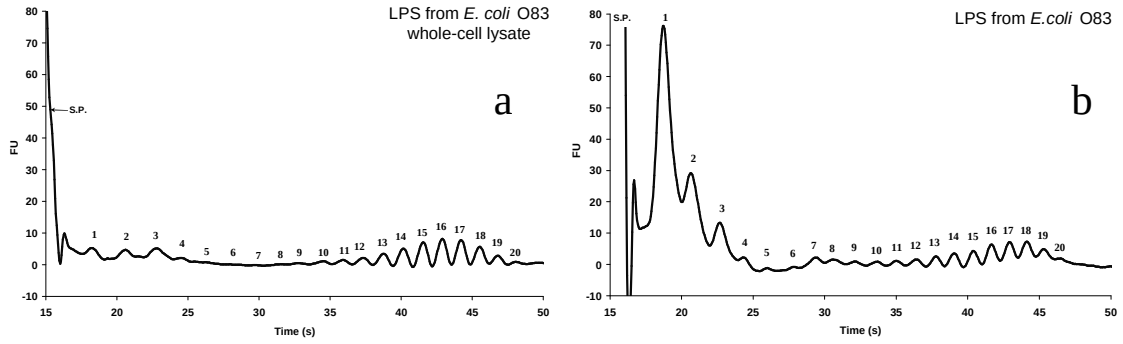

Figure 21. Microchip electrophoretic profiles of a) *E. coli* O83 LPS prepared from whole-cell lysate and of b) pure (1 mg/ml) *E. coli* O83 LPS sample. Experimental conditions were as in Fig. 16d. Electropherograms were qualitatively similar to each other. S.P. = system peak.

The two electropherograms showed great consistency, both having “wave-like”

patterns of *ca*. 20 peaks characteristic for this *S* type LPS. However, the relative peak area ratios were different in the two patterns.

"Wave-like" electropherograms of LPS preparations from whole-cell lysates of five *S* type bacterial strains are shown in Fig. 22.

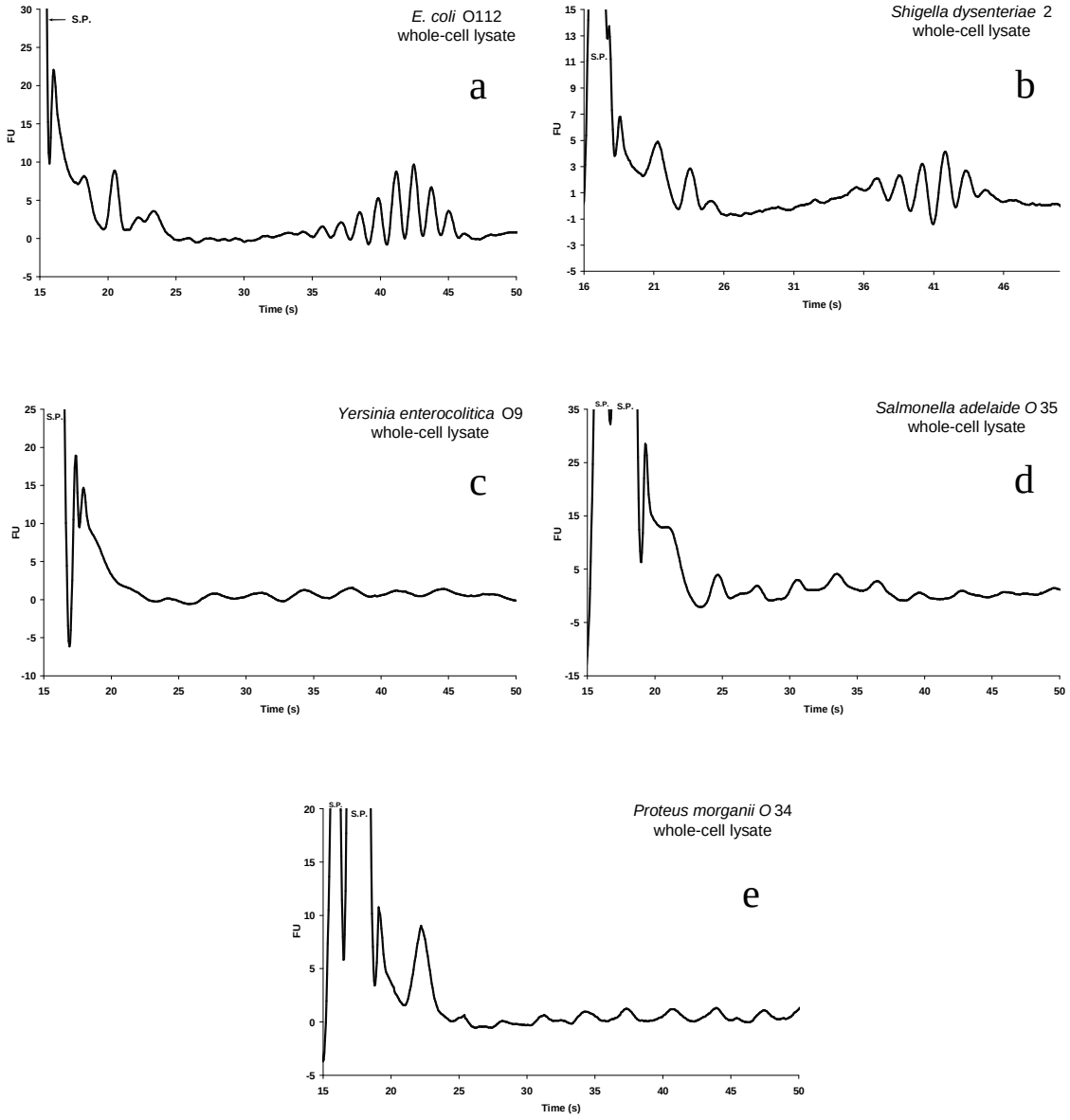

Figure 22. Microchip electrophoretic profiles of *S* type LPS samples prepared from whole-cell lysate of a) *Escherichia coli* O112, b) *Shigella dysenteriae* 2, c) *Yersinia enterocolitica* O9, d) *Salmonella adelaide* O35 and e) *Proteus morganii* O34 strains. The experimental conditions were the same as in Fig. 16d. The peaks after the system peak (S.P.) corresponded to LPS components having O-polysaccharide chains with different lengths.

The patterns detected the presence of several LPS components with various

molecular masses, according to the repeating units in the O-side chains. Both the molecular masses estimated by the chip software and the relative amounts of the components (peaks) showed a characteristic pattern to the respective bacterial origin of the LPSs.

5.3.2. Microchip electrophoresis of *R* type endotoxins prepared form whole-cell lysates

Microchip electrophoresis experiments of LPS prepared from whole-cell lysate of *Salmonella minnesota* R595 bacteria and that of the pure *S. minnesota* R595 LPS are shown in Fig. 23a and b, respectively. The two electropherograms showed the typical pattern of *R* chemotype LPS migrating as a single component closely after the system peak.

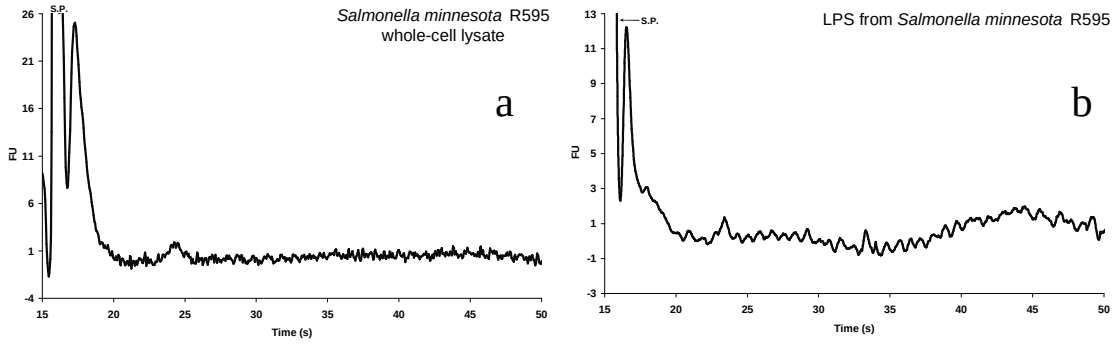

Figure 23. Microchip electrophoretic profiles of a) *S. minnesota* R595 LPS prepared from whole-cell lysate and of b) pure (1 mg/ml) *S. minnesota* R595 LPS sample. Experimental conditions were as in Fig. 16d. Electropherograms were very similar to each other. S.P. = system peak.

Microchip electrophoretic patterns of whole-cell lysates of three *R* type LPSs *Shigella sonnei* 4350, 562H and 41 are shown in Fig. 24. Their profiles indicated that the LPSs were originating from *R* type Gram-negative bacteria, since only one or a few peaks appeared in the electropherograms, with the first peak partially overlapping with the system peak. The migration time was 16.8 s for 4350 LPS and 18.6 s for 562H LPS. In the profile of the 41 LPS two peaks were visible, at migration times 15.55 s and 16.15 s.

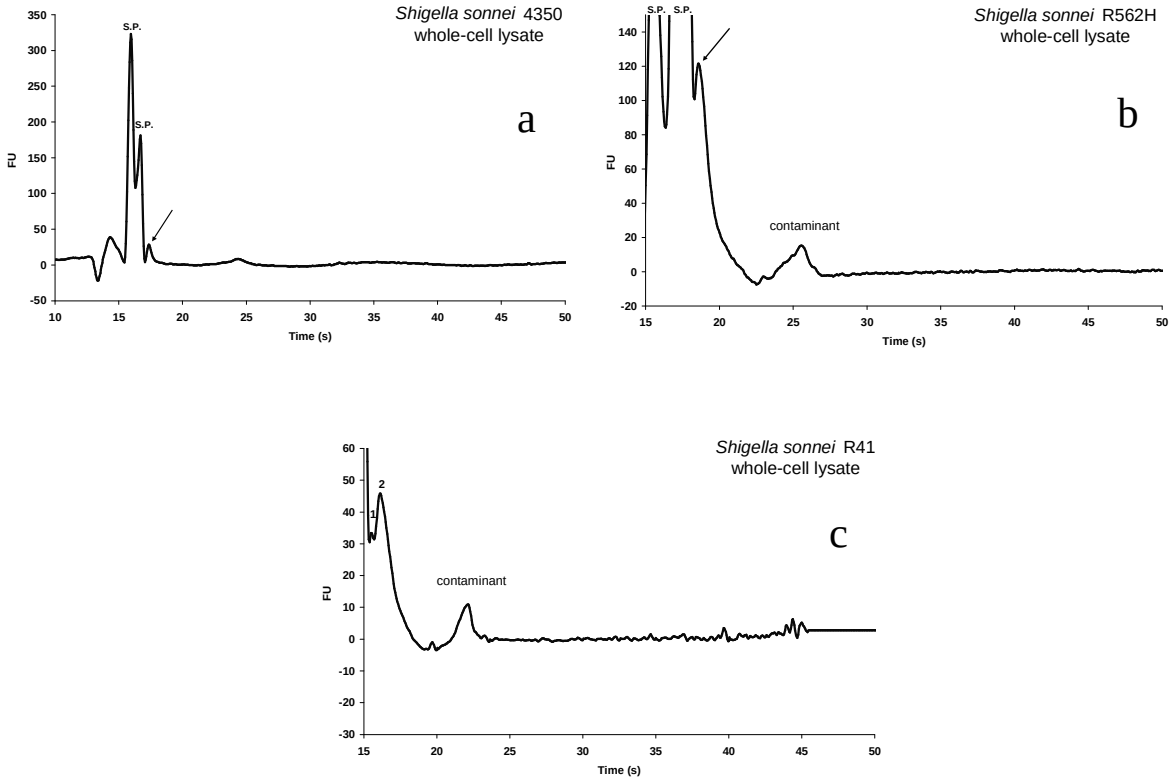

Figure 24. Microchip electrophoretic profiles of *R* type LPS samples prepared from whole-cell lysates of a) *Shigella sonnei* 4350, b) 562H and c) 41 strains. The experimental conditions were the same as in Fig. 16d. The LPS peaks are numbered or indicated by arrows. S.P. = system peaks.

5.3.3. Assignment of endotoxin *S*-*R* chemotypes

Altogether, eighteen partially purified LPS samples were prepared from whole-cell lysates and analysed with microchip electrophoresis. Table 2 shows the classification of the LPSs according to the electrophoretic profiles obtained after blind determination of whole-cell lysate samples. In each case, the classification was proven afterwards with the comparison of the electrophoretic profiles to those of the respective pure endotoxins.

Table 2. Bacterial strains and types of LPSs prepared from whole-cell lysates identified by microchip electrophoresis

Species	Strain	LPS type*
Escherichia coli	O21	*S*
Escherichia coli	O55	*S*
Escherichia coli	O83	*S*
Escherichia coli	O111	*S*
Escherichia coli	O112	*S*
Escherichia coli	D31	*R*
Proteus morganii	O34	*S*
Salmonella urbana	O30	*S*
Salmonella adelaide	O35	*S*
Salmonella minnesota	wildtype	*S*
Salmonella minnesota	R595	*R*
Shigella sonnei	phase I	*S*
Shigella sonnei	phase II (4303)	*R*
Shigella sonnei	41	*R*
Shigella sonnei	562H	*R*
Shigella sonnei	4350	*R*
Shigella dysenteriae	2	*S*
Yersinia enterocolitica	O9	*S*

* *R*: rough-type; *S*: smooth-type

5.4. Endotoxin analysis by MALDI-TOF MS

MALDI-TOF mass spectrometry measurements were performed with intact *R* type endotoxins extracted from *Salmonella minnesota* R595 and *Shigella sonnei* 41 strains, as well as with the intact *S* type lipopolysaccharide extracted from *E. coli* O83 strain. Furthermore, the Lipid A parts obtained by mild acid hydrolysis from *Shigella sonnei* 41 and from *E. coli* O83 LPS were also studied. All samples were suspended in 0.1 M citric acid, decationized with Dowex (NH_4^+) and crystallized on 2,5-dihydroxybenzoic acid (DHB) matrix. Negative ion mass spectra were obtained with an Autoflex II. MALDI-TOF/TOF instrument operating in linear mode for intact endotoxins and in reflectron mode for the Lipid A samples. Masses obtained with linear mode conditions were average masses. Because of the lower resolution power of the TOF analyser in linear mode, the peaks were often broad compared to the measurements with reflectron mode conditions. In reflectron mode, the isotopic patterns of the molecules were resolved, so the mass values determined corresponds to monoisotopic masses.

The molecular masses of the LPS or Lipid A constituents found to be present in the examined endotoxins are given in Appendix 4, and the interpretations of the molecular ions appearing in the spectra, *i.e.*, the composition of the LPS molecular species are given in Appendix 5.

5.4.1. Analysis of the intact (*R* type) *S. minnesota* R595 LPS

The negative ion MALDI mass spectrum of the intact *S. minnesota* R595 LPS is shown in Fig. 25. The spectrum presents several ions as a consequence of both, the intrinsic heterogeneity of this LPS (*i.e.*, the size and hydroxylation of acyl chains on the Lipid A and on the Lipid A and core component of LPS), and the hydrolysis steps induced during "in source" dissociations of the LPS species [Madalinski et al., 2006]. The $[M-H]^-$ quasimolecular ions at *m/z* 1361, 1570 (and also 1597), 1797 and 2035 correspond to the tetra, penta, hexa- and hepta-acylated free Lipid A forms, respectively. The expression "Lipid A form" used here and in the following chapters refers to the basic Lipid A structure consisting of a bis-phosphorylated glucosamine disaccharide, which can be acylated with 3-hydroxylated fatty acids linked as esters at 3, 3' and as amides at 2, 2' positions (primary substitution), along with further fatty acids connected in ester linkage at 3 position of the primary 3-hydroxylated fatty acids (secondary substitution) [Raetz, 1990; Silipo et al., 2002]. The signals at *m/z* 2238 and 2476 correspond to molecular ions containing the hexa- and the hepta-acylated Lipid A and two Kdo molecules ($\Delta m/z = 440$), respectively. Ions at *m/z* 2166, 2369 and 2607 carry an additional residue of an aminopentose, 4-amino-4-deoxy-L-arabinose (L-Ara4N, $\Delta m/z = 131$) [Boll et al., 1994] with respect to *m/z* 2035, 2238 and 2476. A possible interpretation for the ion at *m/z* 2035 is a penta-acylated Lipid A form (for which *m/z* is 1597) carrying two Kdo molecules.

According to this mass spectrum, the structure of this LPS can be proposed as shown in Fig. 26. In details, a bis-phosphorylated glucosamine disaccharide carrying four residues of 3-hydroxytetradecanoic acid (3-hydroxymyristic acid, C14-OH) at positions 2, 3, 2', 3′, of which three are acylated at the 3-hydroxy groups by one dodecanoic acid (lauric acid, C12), one tetradecanoic acid (myristic acid, C14) and one hexadecanoic acid (palmitic acid, C16), respectively, and to which two Kdo molecules are linked at 6', as well as an additional L-Ara4N constituent of the ester-linked phosphate-group at position 4'.

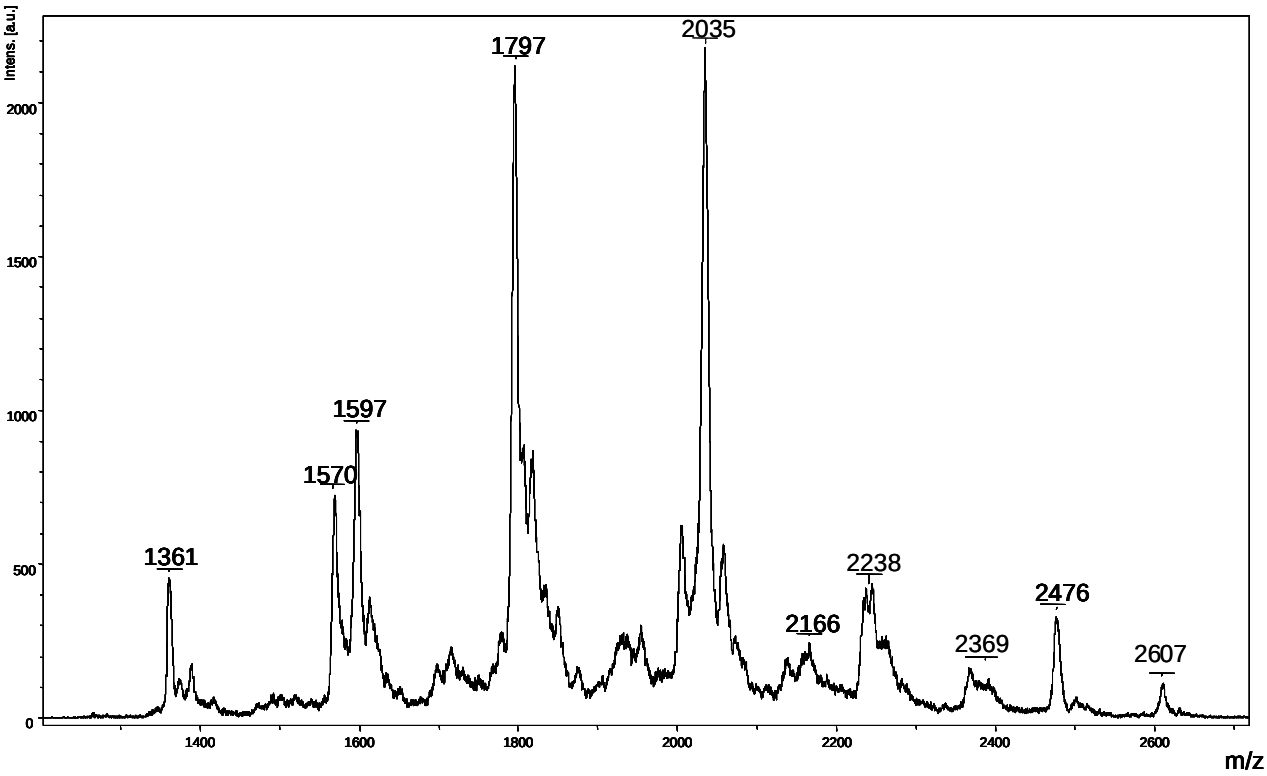

Figure 25. Linear mode negative-ion MALDI-mass spectra of LPS extracted from *S. minnesota* R595 strain. The relevant ion peaks are described in the text and in Appendix 5.

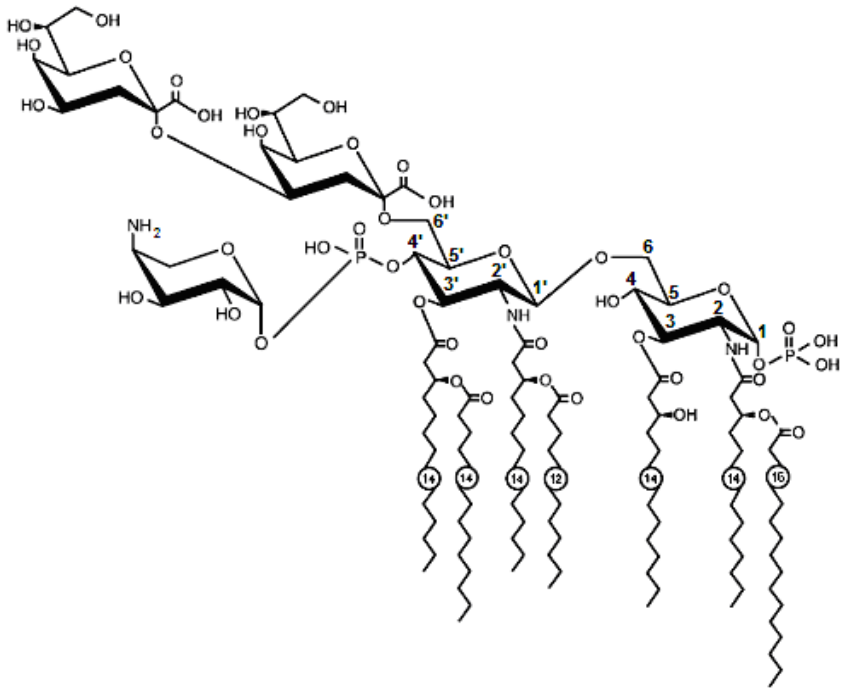

Figure 26. Proposed structure of the intact *S. minnesota* R595 LPS according to its mass spectrum in Fig. 25 and in accordance with the previous studies [Brandenburg et al., 2003]. For detailed structural description see also text.

5.4.2. Analysis of the intact (*R* type) *S. sonnei* 41 LPS and its Lipid A

The negative MALDI-TOF mass spectrum of the Lipid A of *S. sonnei* 41 LPS (Fig. 28a) showed ion peaks that could be identified as follows: the molecular ion at *m/z* 1797 corresponds to a hexa-acylated Lipid A form containing four hydroxy-myristic acid (C14-OH), one myristic acid (C14) and one lauric acid (C12) residues; the ion at *m/z* 1569 can be explained with the absence of a C14 secondary fatty acid through ester bond cleavage from an ester linked primary (C14-OH) fatty acid, producing an unsaturated C-14 (C14unsat) ester side chain; the ion at *m/z* 1361 account for the additional lack of a C14-OH residue; ions at *m/z* 1717 and 1281 are monophosphorylated species of the hexa-acylated Lipid A ion at *m/z* 1797 and the tetra-acylated one at *m/z* 1361, respectively. The peak at *m/z* 1489 is the corresponding monophosphorylated Lipid A form of that at *m/z* 1569 and ion at *m/z* 1054 is the monophosphorylated tri-acylated Lipid A form with two C14-OH and one C12 fatty acyd constituents.

In the spectrum of the intact LPS (Fig. 28b) some additional peaks can be seen attributable to tri-acyl (at *m/z* 1959), tetra-acyl (at *m/z* 2185), penta-acyl (at *m/z* 2394) and hexa-acyl (at *m/z* 2622) LPS molecular species, consisting of the respective Lipid A forms carrying two Kdo and two Hep substituents, since the difference for this core composition ($\Delta m/z = 825$) is also the difference between the respective mass signals. The peak at *m/z* 2087 is the corresponding monophosphorylated LPS species of that at *m/z* 2185 (with $\Delta m/z = 98$) and ions at *m/z* 2265, 2473 and 2703 are the corresponding triphosphorylated LPS ions (with $\Delta m/z = 80$) of the tetra-acyl, penta-acyl and hexa-acyl bis-phosphorylated LPS species, respectively. This additional phosphate group could be a substitutent either of a Kdo residue or a heptose residue. The peaks of the intact triphosphorylated LPS species are also present as sodium adducts (with $\Delta m/z = 23$) (peak data are not shown). The proposed structure of this LPS, consisting of a bis-phosphorylated diglucosamine bearing six fatty acids (four C14-OH, one C14 and one C12) connected to a core region composed of two Kdo and two Hep molecules and an additional phosphate group, is shown in Fig. 29.

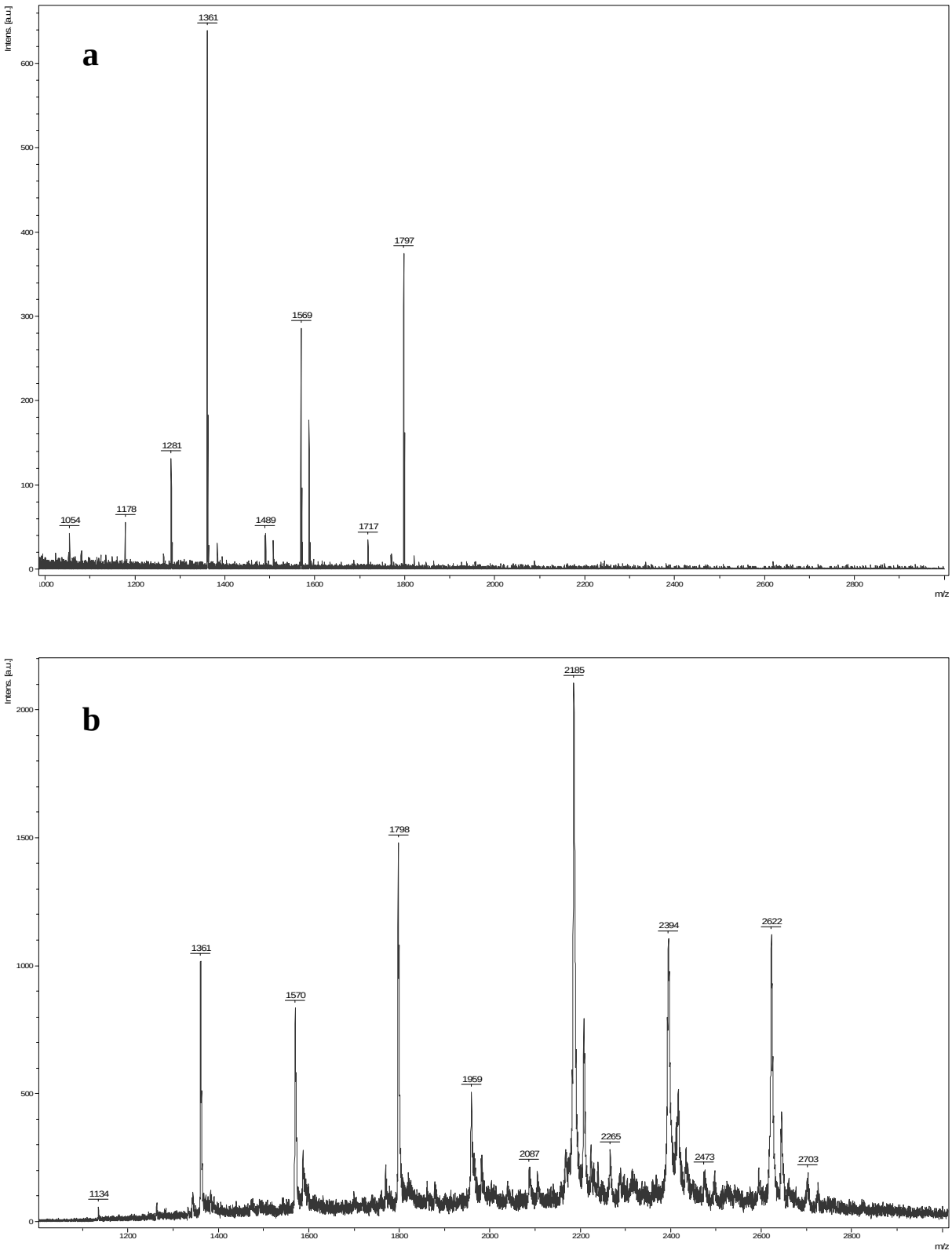

Figure 28. Negative-ion MALDI-TOF mass spectra a) of the Lipid A from *S. sonnei* 41 LPS measured in reflectron mode and b) of the intact *S. sonnei* 41 LPS measured in linear mode. The relevant ion peaks are described in the text and in Appendix 5.

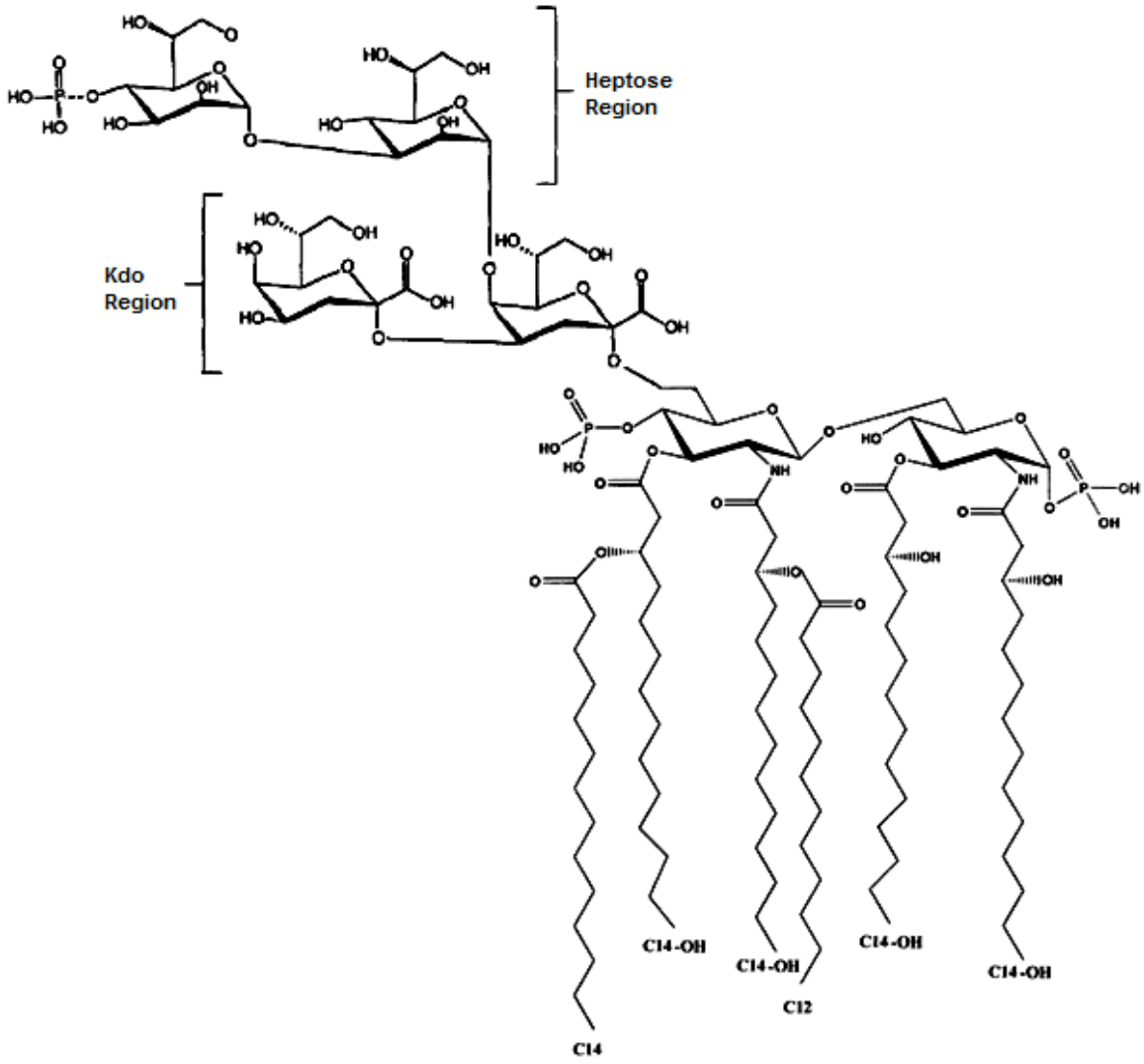

Figure 29. Proposed structure of the intact *S. sonnei* R41 LPS according to its mass spectrum in Fig. 28. For detailed structural description see also text.

5.4.3. Analysis of the intact (*S* type) *E. coli* O83 LPS and its Lipid A

The negative MALDI-TOF mass spectra of *E. coli* O83 Lipid A (Fig. 30) contains the same major and minor species at signals *m/z* 1797, 1717, 1569, 1361 and 1281, which have been identified in spectrum of *S. sonnei* 41 Lipid A (compare Figs. 28b and 30). The ion at *m/z* 1587 could be explained with the absence of a secondary fatty acid C14 from the hexa-acylated Lipid A form at *m/z* 1797, and the ion at *m/z* 1134 could be described by the presence of a secondary fatty acid C12 on a primary C14-OH in the tri-acylated residue. The ions at *m/z* 1826 and 1769 indicate some microheterogeneity in the fatty acid distribution, because they correspond to bis-phosphorylated hexa-acylated Lipid A species

containing four C14-OH, one C12 and one C16 residues, as well as four C14-OH and two C12 residues, respectively. Traces of the bis-phosphorylated hepta-acylated Lipid A form at *m/z* 2036 containing an additional palmitic acid (C16) linked to a primary C14-OH is also found. The proposed structure for this Lipid A is presented in Fig. 31.

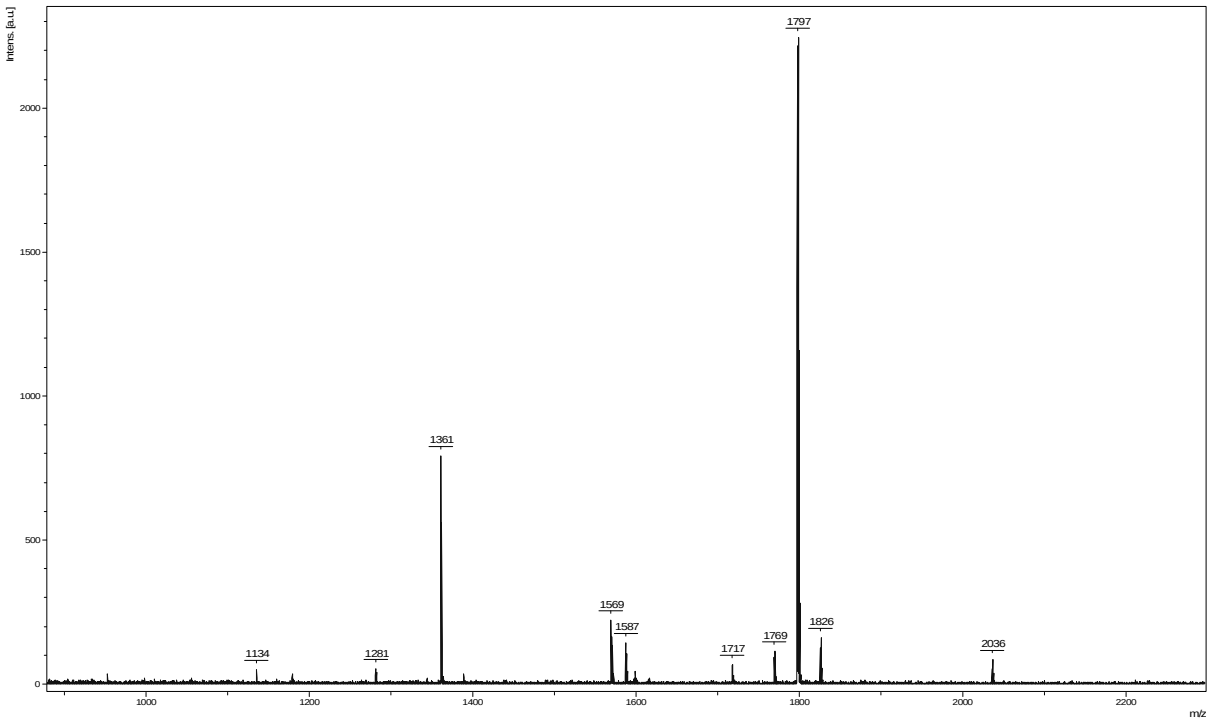

Figure 30. Negative-ion MALDI-TOF mass spectrum of Lipid A from *E. coli* O83 LPS in reflectron mode. The ion peaks are described in the text and in Appendix 5.

The negative ion MALDI mass spectrum of the intact *E. coli* O83 LPS revealed a complex pattern of ions representative of about five distinct mass regions. The lower mass region between *m/z* 1000 – 2000 Da seen in Fig. 32 corresponds to Lipid A species with different acylation and phosphorylation degrees (see their interpretations in Appendix 5). The next group of peaks between *m/z* 3400–4400 correspond to the sum of the Lipid A and core LPS components, *i.e.*, to O-chain-free LPS molecular ions. The approximate mass of the core was given by the difference between *m/z* 3868 and 1826 (the molecular ion of the Lipid A), calculated as $\Delta m/z = 2042$. This mass difference could account for the common core structure of *E. coli* strains composed of three Kdo, three Hep, three Glu and two Gal residues (the sum of these molecules of which is 2046 Da, see masses of the sugars in Appendix 4).

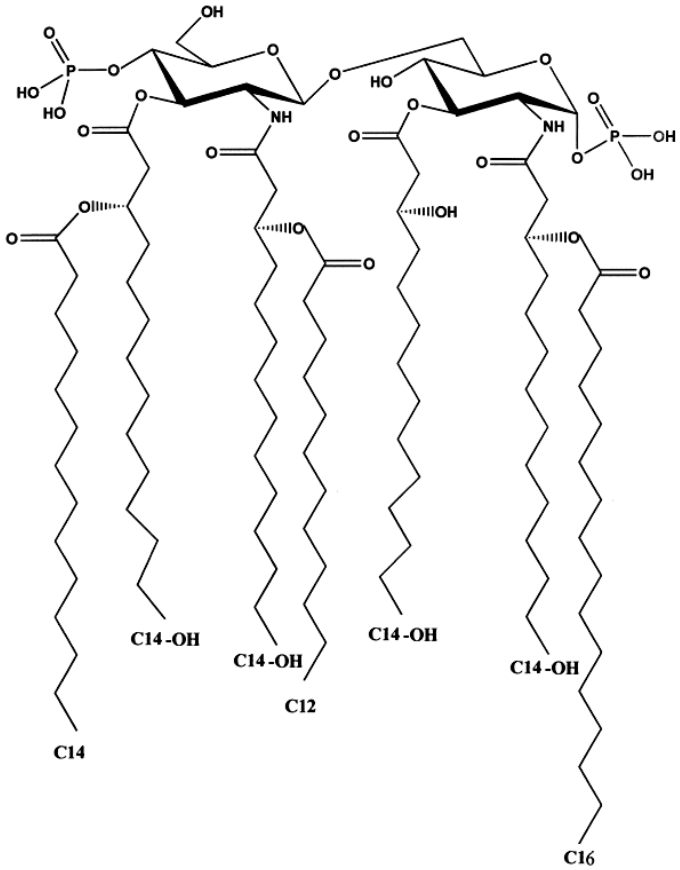

Figure 31. The structure of *E. coli* O83 hepta-acylated Lipid A according to the ions occurring in the MALDI-TOF MS spectrum in Fig 30.

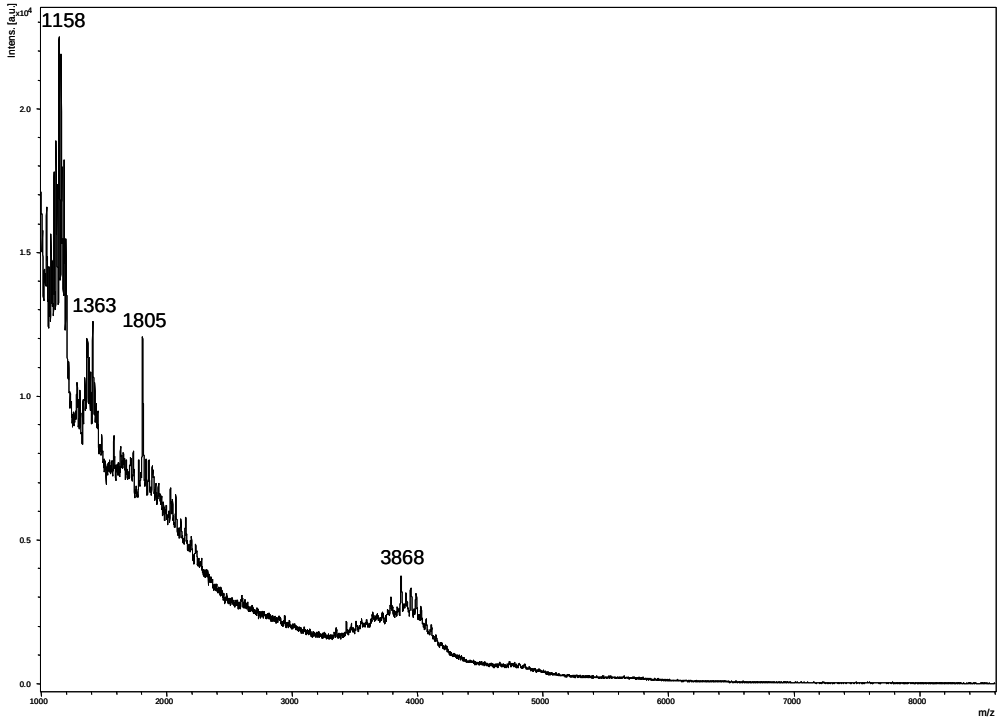

Figure 32. Linear, negative-ion mode MALDI-mass spectra of LPS from *E. coli* O83 measured between *m/z* 1000 – 10000.

The fine structure of the second mass zone is shown in Fig. 33, in which the mass difference between the peaks was about 80 Da, corresponding to a phosphate group present in the LPS molecular species. This suggests that heterogeneity is a result not only due to LPS components with differences in the sugar constituents and the acyl chains, but also due to the diverse phosphorylation states of the Lipid A and the core.

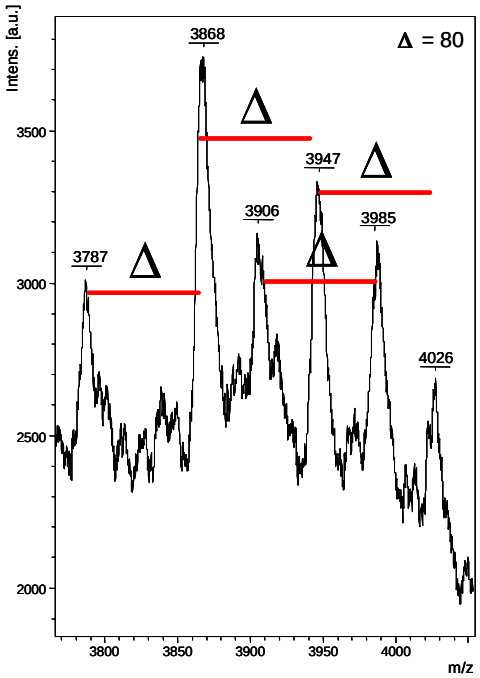

Figure 33. Fine structure appearing in the mass spectrum of LPS extracted from *E. coli* O83, showing differently phosphorylated molecular species (mono-, bis-, tris-phosphorylations, *etc.*) consisting of Lipid A and core and one repeating unit. $\Delta m/z = 80$ reflects a phosphate group.

The *E. coli* O83 LPS sample was furthermore measured by MALDI-TOF with the mass interval adjusted to be between *m/z* 2000 and 20000. The mass spectrum is showed in Fig. 34a, in which three regions can be seen between *m/z* 4600 and 5000, *m/z* 5400 and 5800, and between *m/z* 6200 and 6700. The signals at *m/z* 3868, 4734, 5600 and 6465 are attributable to molecular ions of LPS glycoforms, with differences of 866 Da. This difference correlates to one oligosaccharide repeating unit in this LPS [Jann et al., 1994], consisting of one Glu, two Gal, one GluN and one GluA unit (see molecular masses of these molecules in Appendix 4). We assume that these ions were the most ionizable intact LPS molecular components giving signals in the mass spectrometer. Since these four masses correspond to LPS species carrying 0, 1, 2 and 3 repeating units, these regions are consistent with the first four peaks, respectively, found in the microchip profile of *E. coli* O83 LPS (Fig. 34b).

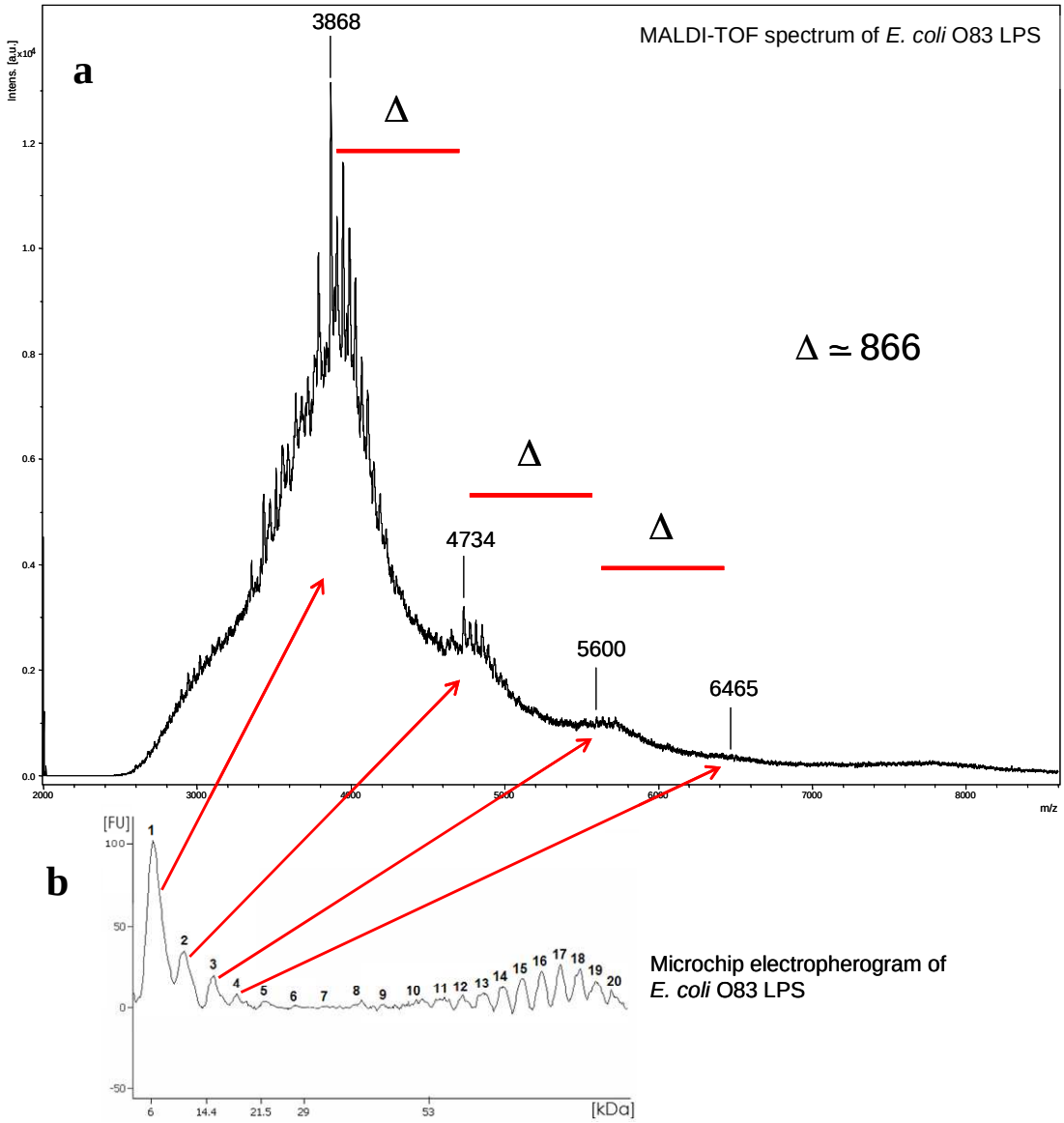

Figure 34. a) Negative-ion MALDI-mass spectra of LPS from *E. coli* O83 (measured in linear mode between *m/z* 2500 and 10000) in relation to b) the microchip pattern of the same LPS. The first peak in the microchip electropherogram corresponds to the Lipid A and core species of the LPS, while the following peaks appear with differences equal to mass differences of the O-side chain repeating unit (Δ*m/z* = 866 Da).

From the number of peaks in the microchip electropherogram (Fig. 34b) it was possible to deduce the number of repeating units present in the major molecular species of this LPS to be 19 repeating units (see also chapter 5.2.1). The masses of *E. coli* O83 LPS components were calculated as the sum of the masses of its Lipid A (1826), core (2048) and O-chain repeating units (866) multiplied by 1 to 19. The highest molecular mass assuming 19 repeating units is consequently 20328 Da.

6. Discussion

The bioactivity of endotoxins is principally determined by their primary structure, thus the knowledge of the chemical composition of the bioactive parts of endotoxins from individual strains is important. The characterization of endotoxins typically involves the combined use of methods, since one analytical technique *per se* is not able to identify LPSs, to determine the molecular structures, to provide data on molecular masses, and to characterize the relative abundance and structural heterogeneity of each LPS glycoform. To date, the separation of glycoforms of the native LPS is routinely performed by SDS-polyacrylamide gel-electrophoresis method with silver staining [Tsai and Frasch, 1982]. However, the silver stain detection has limited sensitivity since the visualization often results in doublets or diffuse/broad staining with poor resolution rather than distinct LPS bands. This is basically the result of an incomplete dissociation of LPS aggregates during electrophoresis [Hitchcock, 1983]. Despite that several improved versions of this technique exist, including pre-electrophoresis step [Hitchcock, 1983], concentration gradient gel [Inzana and Apicella, 1999; Noda et al., 2000], use of increasing amount of SDS [Peterson and McGroarty, 1985] or replacement of SDS by sodium deoxycholate (DOC) [Komuro and Galanos, 1988], SDS-PAGE has some major drawbacks being labor-intensive, time-consuming, difficult to automatize and to standardize, and the quantification (made by densitometry) is practically complicated.

In our studies, new, fast and sensitive electrophoretic methods – both capillary and microchip electrophoresis – were introduced for the detection and quantification of endotoxins extracted from various bacterial strains of the *Enterobacteriaceae* family. Characteristic and reproducible profiles were recorded for the bacterial strains and their genetic variants, and the profiles were suitable for comparative analysis, as well. Above pH 2.3 the LPS molecules are negatively charged owing to the phosphate residues on the Lipid A part and the carboxyl groups in the Kdo molecules. Capillary electrophoresis using a simple buffer (Tris-HCl, pH 8.5) was applied to detect endotoxins by complexation of relatively fast migrating proteins, such as hemoglobin (Hb) and transferrin (Tf). LPSs do not absorb light in the UV region and could, therefore, be detected only as complexes with either of these proteins, by their UV-absorption. The mechanism of the binding is unclear, although the hypothetical function is possibly a disaggregation of lipopolysaccharide micelles by the protein, followed by the interaction

between the two molecules by electrostatic and hydrophobic interactions [Berger et al., 1991; Roth et al., 1996].

Human hemoglobin is a globular oxygen-carrying 64-kDa protein consisting of four-subunits (two α and two β peptide chains), each carrying a heme moiety, that is a ferrous Fe^{++} iron-containing porphyrin. Based on the well known fact, that native human hemoglobin forms stable and soluble complex with endotoxins with dissociation constant of 3.1×10^{-8} M [Kaca et al., 1994], the applicability of this protein was demonstrated to the analysis of LPSs from *Salmonella minnesota, Shigella sonnei* and *Escherichia coli* strains (Figs. 9–12). Through LPS-Hb complexes it was possible to distinguish between LPSs with significant differences in their structure, thus, Hb could be used as a marker of rough and smooth type of endotoxins. The changes in the spectral properties of Hb upon binding of LPS were a proof of the complex formation (Fig 13). Although, LPS can accelerate oxidation of Hb to methemoglobin, *i.e.*, the heme irons in the subunits of Hb are oxidized to the ferric Fe^{+++} [Jürgens et al., 2001; Currell and Levin, 2002], no essential changes in the Hb secondary structure was found, as indicated by IR-spectroscopy measurements [Kaca et al., 1995].

It has been reported that endotoxin contamination in artificial hemoglobin can result in side effects, therefore, this detection method could be of importance from the aspect of hemoglobin, as well. Accurate measurement and effective elimination of endotoxins are important in producing safe hemoglobin preparations. The newly developed CE method for detecting Hb-endotoxin complexes could control the preparative process of artificial blood and might be an advanced method in the quality control of such samples. It has been reported that each chain of the Hb tetramer might bind a single smooth LPS, or 3–5 rough LPSs [Jürgens et al., 2001]. Although electrophoresis cannot easily be used for such determinations it was likely that not all smooth endotoxin molecules from *S. minnesota* wildtype in the HB-LPS mixture solution were bound to Hb, since the overlapping complex peak (next to the free Hb peak) seen in the electropherogram (Fig. 11) did not change upon longer incubation. From the molar ratio of the smooth LPS to Hb we could assume that probably not more than 1-2 LPS molecules bound to each protein molecule. In the cases of the *R* type endotoxins (of much higher LPS/Hb molar ratio) a significant amount of the LPSs should be bound to Hb. The “disappearance” of the free Hb peak upon prolonged incubation with LPS from *S. sonnei* 562H (Fig. 10b) indicated that the protein molecules bound several molecules (up to 20) of this *R* type LPS at the same time. The complex formation of the major and minor Hb components with endotoxins was

different, since the ratios of these components in the complexed forms and in the free forms varied. From the electropherograms, however, it was not possible to conclude whether this phenomenon was due to a difference in the binding strength of the different Hb components or due to other effects. LPSs form different strains of *E. coli* and *S. sonnei* bacteria formed complexes with Hb of different mobility values and peak shapes (Fig. 12). Consequently, the different *S* or *R* type LPSs could be differentiated by this method, although, with poor resolution.

For LPS-protein interactions transferrin (Tf) was also studied by CE, since human serum transferrin is reported to be another major lipopolysaccharide binding protein of human plasma [Berger and Beger, 1987], but with unknown dissociation constant. Tf is an iron-delivering, single chain glycoprotein of approximately 77 kDa, and in fact, it is a mixture of Tf isoforms, also called sialoforms due to microheterogeneities of sialic acid residues. These isoforms can be resolved into 1-6 peaks in CE under optimum experimental conditions using a buffer consisting of 18 mM boric acid and 0.3 mM EDTA (pH 8.4), 8 kV separation voltage and capillary i.d. of 0.1 mm [Kilár and Hjertén, 1989]. In our separation system (using a 50 mM Tris-HCl buffer solution at pH 8.5, capillary i.d. 0.05 mm, and voltage of 10 kV), the electrophoretic pattern of the free Tf isoforms could be resolved into 3 broad peaks only (Fig. 14a). Consequently, the detectability of the Tf-LPS complex peaks were also affected (the peaks were not enough sharp, see for instance Fig. 14b). However, the separation patterns detected at 205 nm were similar with Tf and Hb following complex formation with LPS from *S. minnesota*, *S. sonnei* and *E. coli* strains, indicating that the LPSs qualitatively interacted similarly with the two proteins (compare Fig. 10b with 14b, 9c with 15a, and 12b with 15b).

Main advantages of this CE method [Kilár et al., 2006] were the rapid separation (profiles were obtained within 20 minutes), the simplicity and the demonstration of the presence of known LPS in solution. Furthermore, by the determination of the electrophoretic mobilities, we found this method useful to observe qualitative differences in LPSs expressed by different bacterial strains. Since the endotoxins showed different behaviour during complexation with the proteins, only a rough estimation of the detection limit could be given. The lowest concentration of the major complex peak detectable of the rough LPSs was 50 μg/ml.

An LPS molecule usually contains two phosphate groups with pK_1 value around 1.3 [Hirayama and Sakata, 2002], a strongly hydrophobic group (the fatty acids) and a hydrophilic polysaccharide part. The LPS molecules can, thus, take part in electrostatic

and hydrophobic interactions with surface agents, for instance SDS [Olins and Warner, 1967; Hitchcock, 1983]. In this work, a new analytical method for monitoring LPSs is also presented based on the realization that endotoxin–SDS complexes (or rather complexes of endotoxins and dodecyl sulfate chains) can be visualized by appropriate fluorescent dyes, which form complexes with the dodecyl sulfate chains. For this, the routine analysis developed by Bousse *et al*. (2001) was modified and adapted to detect protein–SDS complexes in chip-electrophoresis. Since the SDS content is not known in the commercially available sample buffer for protein analysis, and also, because it contains other components, which might disturb the analysis, SDS–water solution is used for the disaggregation of the LPSs. The effect of the SDS concentration on the solubilization of the LPS molecules was studied and the LPS aggregates were satisfactorily dispersed in SDS solutions with at least 40-50 mM dodecyl sulphate concentration (Fig. 16). LPS patterns were then visualized as, both, electropherograms and gel-like patterns by the chip software and reflected the same size heterogeneity as observed in the SDS-PAGE ladder-patterns (Fig. 20).

The electrophoretic separation of LPS components of the *S* type endotoxins from *E. coli* and *S. adelaide* strains show a "wave-like" profile (Figs. 16d and 18). The electrophoretic migration differences of these *S* type endotoxins are attributed to differences in their chemical composition, *i.e.*, the monosaccharides present in the core part, and the composition and number of repeating units in the O-polysaccharide chains. We assume that the first peak in the electropherograms corresponds to Lipid A and core part (*i.e.*, the "*R*" component of the *S* type endotoxin species), and the remaining peaks reflect the number of O-chain repeating units. In the profiles of the *R* type LPSs of *S. minnesota, E. coli* and *S. sonnei* strains, one single or very few peak components are present (Fig. 17), probably because these were mutants, which synthesized endotoxins without O-side chains and sometimes without portions of the core, consequently, they lack the "wave-like" profile. In the microchip, one major LPS component (peak) of the *S. minnesota* R595 endotoxin is resolved (Fig. 17a). Since this peak is rather broad, it is most probably composed of differently acylated LPS molecular species, *i.e.*, a mixture of LPS molecules containing a Lipid A part carrying different number of fatty acids. According to the mass spectrum in Fig. 25 (and as earlier described by Brandenburg et al. in 2003) we presume that the major species of R595 LPS contain a hepta-acylated Lipid A part linked to a core part consisting of two Kdo and one L-Ara4N residues. The two components (peaks) of *S. sonnei* 41 (Fig. 17c) are assumed to correspond to differently acylated LPS

molecules, as well. Deducing from the mass spectra (Fig. 28) of this LPS, we assume that the *S. sonnei* Lipid A part bearing two Kdo-s, and the Lipid A part linked to the complete core part (with additional two Hep-s) are the major molecular species corresponding to the first and the second peak, respectively. The compositions of the components (peaks) in the microchip eletrophoretic profile of *E. coli* D31 and *S. sonnei* 4303 (Fig. 17b and d) are not known. The first peak for the *R* type endotoxins several times overlapped with the system peak but they are reproducible peaks and do not appear in the absence of LPS (*i.e.*, in blank samples).

The unknown endotoxin of *E. coli* 102 strain is obviously a smooth type LPS with the characteristic "wave-like" pattern (Fig. 18b). Although, our microchip technique is suitable for the detailed quantitative analysis of the LPS species, the correct molecular mass determination is possible only by using appropriate internal standards, which is not available yet. Even the existing SDS-polyacrylamide gel electrophoresis method, which gives good resolution, suffers from the lack of appropriate molecular-mass standards. However, the quantitative evaluation of the relative amounts of the different LPS components (peaks) by integration of the microchip electropherograms is a leap forward. Table 1 shows that the theoretical molecular mass values for *E. coli* O83 LPS calculated from the chemical structure according to Rietschel et al. (1994) and Jann et al. (1994), along with our mass spectrometric measurements presented in Fig. 30, and those estimated by using the migration properties of the protein molecular mass standards, provides inconsistent values. However, this is obvious, since a LPS has significantly different chemical properties from a protein, thus, the LPS–dodecyl-sulphate complexes are formed with unknown ratio, to which, in addition unknown amount of flurescent dye binds in the microchip during the run. This result in a net surface charge of the LPS–SDS–dye complexes substantially different from that of the SDS-denatured proteins, consequently, the migration properties of the two types of complexes are different. Therefore, it is not surprising, that the LPS molecular mass values estimated by using a protein mass standard (either in SDS-PAGE or in microchip) do not suit to their calculated mass values.

Structural analyses of endotoxins are usually performed on samples obtained from large-scale preparations and long-lasting (5 to 10 days) purification processes [Westphal et al., 1952; Galanos et al., 1969] requiring appreciable amount of bacteria. However, when large number of bacterial mutants and their LPS content are to be compared (for instance in the preparation of vaccines, or for O-serotyping of LPSs in epidemiological

studies) endotoxins are prepared directly from bacterial cultures by the relatively short method of Hitchcock and Brown (1983). This method involves the treatment with lysozyme followed by the proteolytic digestion of whole-cell lysates by proteinase K resulting in protein-free LPS without further purification steps and freeze drying (hence it is called partially purified LPS). An important speciality of this method is that as small as 1 ml culture volume (cultured from one spot of bacterial colony grown on agar plate) is enough for the preparation of the LPSs, with overall process-time of *ca.* 40 h.

In this work, we showed that our novel microchip method [Kilár et al., 2008a] is applicable for the fast detection of endotoxins after extraction of the partially purified samples obtained from careful small-scale preparations of bacterial whole-cell lysates [Kilár et al., 2008b]. The electrophoretic profiles of 18 different endotoxins in bacterial lysate samples were successfully used for the proper assignation of the LPS chemotypes (Table 2). Each of the eighteen endotoxins was characterized from cell lysate without fault, which was approved with endotoxin profiles of purified LPSs, and the LPS chemotypes had unique migration profiles, as expected. The *R* chemotypes contain high-mobility (low-molecular-mass) components presumably consisting of Lipid A and core oligosaccharide (Figs. 23 and 24). The LPSs of *S. sonnei* strains 4350, 562H and 41 are isogenic mutant variants containing the same Lipid A part, but different core regions [Kocsis and Kontrohr, 1984; Kontrohr and Kocsis, 1981]. The differences between these three *R*-type LPSs could also be followed in the migration properties of the respective whole-cell lysate samples (see the profiles in Fig. 24). The *S* type LPSs possess components with low-mobility (high-molecular-mass) and appear as “waves” of peaks according to the number of repeating units in the O-side chains (Figs. 21 and 22). However, it should be stressed that the electropherograms of the LPSs in the whole-cell lysates were in some cases slightly distorted compared to the profiles of the respective pure LPSs. This was observed, for instance, when the relative peak area ratios were different (compare Figs. 18a with 22d, and 21a with 21b), or when some cellular contaminants were found (for instance in Fig. 24b and c), certainly due to differences in the extraction and purification processes.

The sensitivity of this method is high, since satisfactory patterns are obtained using 1 ml bacterial cultures, which contain *ca.* 10^8 cells with an LPS content less than 1 nanogram (observe that the actual injection mass of the LPSs is even smaller). With the help of this microchip analysis 10 endotoxin assignations can be performed within *ca.* 1 h

compared to the SDS-PAGE methodology, which needs a laborious gel-preparation and silver staining for visualization, although, 18–20 samples can be determined in one run.

Studies with endotoxins include another very important and highly challenging area. This is due to the chemical microheterogeneity of the LPS molecules, not only due to the differences between bacterial sources, but also the possible substitutions (*e.g.*, ethanolamine residues, acetyl- or phosphate groups) and variations of the sugar and lipid constituents within one certain species. For the analysis of these intrastrain microhetegogeneities (some of which were seen already in the electrophoretic profiles, as well) appropriate instrumental approaches are necessary.

In this study, we performed MALDI MS measurements, but of course, other MS techniques or NMR studies would also be good choices. The MS studies require the use of LPSs as free acids by replacing the alkali counter-ions (Ca^{2+} and Mg^{2+}) of the acidic groups by a pre-treatment with a very small amount of Dowex cation-exchange resin in its ammonium form. This allows gas-phase proton transfer reactions and facilitates the analyte detection as $[M–H]^-$ in negative ion mode. The use of the saturated solution of 2,5-dihydroxybenzoic acid (DHB) as a MALDI matrix, minimizing the occurrence of prompt fragmentations, is of considerable importance for the analyses of *R* type LPSs, since these molecules undergo laser-induced fragmentation even in „mild" conditions (soft ionization). The use of citric acid increases the solubility of endotoxins considerably, since samples in water gave poor peak resolution (not shown). We found that MALDI-TOF mass spectrometry can successfully be used in the analyses of intact endotoxins and their Lipid A parts (obtained by mild acid hydrolysis). The evaluation of the mass spectra with the help of previously described detailed structural interpretations of analogous mass spectral values of Lipid A parts [Silipo et al., 2002; Madalinski et al., 2006] and proposed core structures [Raetz, 1990] resulted in compositional analyses and proposals for LPS structures from *S. minnesota* R595, *S. sonnei* 41 and *E. coli* O83 bacteria (Figs. 26, 29 and 31, respectively). It should be noted, however, that the mass spectra of the intact *S* type *E. coli* O83 LPS is quite complicated. We could only determine the relative abundances and the molecular masses of the most ionisable components of this LPS. The mass spectrometric measurements along with microchip electrophoretic analysis of the *E. coli* O83 LPS can be used to deduce the structures and the molecular masses of the "low-molecular-mass LPS molecular species", but further studies are required, for the structural evaluation of the molecular species with higher number of the repeating units.

7. Conclusions and future perspectives

We conclude that both, capillary electrophoresis (CE) and microchip electrophoresis can now be applied for endotoxin analysis in the field of bacteriology. These novel techniques provide standardizable, fast, and sensitive methods for the structural evaluation of LPS molecules, especially for the differentiation of the smooth and rough endotoxins. Detection of bacterial lipopolysaccharides is important in both, the identification (chemotaxonomic classification) of bacteria and characterization of mutants.

The CE analysis of endotoxin–protein complexes is a new solution for the visualization of endotoxins in separation methods [Kilár et al., 2006], however, further studies are required to test the sensitivity of the method, *i.e.*, to clarify the migration properties of endotoxins with small structural differences.

Our newly developed microchip technique [Kilár et al., 2008a] made the identification of bacterial chemotypes simpler, faster and more sensitive, compared to the commonly used SDS-PAGE with silver staining. To our knowledge, no detection methods based on gel-electrophoresis in capillaries were used to study endotoxins earlier. Overall, the advantages of this microchip technique are:

i) high sensitivity – since less than a few ng of LPS could be analysed;
ii) high speed and simplicity – since sample preparation (for the microchip analysis) and analyses of 10 different samples are carried out within 1 h;
iii) the requirement of small amount of samples and reagents – picoliters are injected in the chip;
iv) specificity – it is suitable for fast fingerprinting of LPS chemotypes (*R* or *S*) allowing the complete and high resolution separation of LPS glycoforms;
v) quantification – the determination of the relative amounts of the LPS components is possible;
vi) high resolution – since in the routinely used SDS-PAGE with silver staining it is difficult to identify the intensity and exact sizes of the LPS bands.

Future studies involve the elimination of the presence of the system peak in the microchip electrophoretic profile (since this peak impeds the quantitative evaluation of the *R* type LPSs). I should note that after finishing the above studies we were able to develop another novel microchip method, which possesses a new direction in the detection of

endotoxins by covalent labelling of the molecules. Another very important area in endotoxin studies is the finding of appropriate LPS molecules with known chemical structures, which might serve as molecular mass standards for the electrophoretic estimation of the molecular mass of LPSs. Furthermore, it would be interesting to clarify how the complex formation with SDS and fluorescent dyes might influence the migration of the LPSs during electrophoresis, and also to study the possible interaction of fluorescent dyes with the LPS molecules.

Since MALDI-TOF and other MS techniques provide information on the heterogeneity of LPS or LOS preparations, the mass spectrometric investigations can help to study the structure–function relationship of bacterial endotoxins. We believe that the microchip electrophoresis technique together with mass spectrometry measurements will open the way to new achievements on the analysis of LPSs in the field of microbiological examinations.

Appendices

Appendix 1. LPS extraction methods

a. Phenol-water method of Westphal et al. for the extraction of *S* type LPSs

Dried bacteria (50 g) were suspended in 45 ml of distilled water, poured into a blender containing a mixture of 110 g phenol and 65 ml distilled water and stirred for 8 minutes. During this period most of the cells disintegrated and the nucleoprotein-LPS complex was hydrolyzed. The mixture was cooled to 20°C and centrifuged at 4°C (3000×*g*, 10 min). The clear supernatant upper phase was siphoned off and the lower (phenol) phase containing the proteins was extracted with 30 ml of distilled water and centrifuged again. A second 50 g portion of bacterial cells was similarly treated (hydrolytic extraction with phenol–water dialysis, and differential centrifugation). The upper phases were combined and centrifuged again (3000×*g*, 20 min). The combined upper phases were then placed in cellophane bags and dialyzed for 24 hours at 4°C against 28 l distilled water that was stirred continuously and changed occasionally. The dialyzed supernatant was centrifuged (100 000×*g*, 1 h). The LPS sedimented in the form of gel-like transparent material. The sediment was dissolved in 45 ml distilled water per 100 g of wet cells and centrifuged (2000×*g*, 10 min). One hundred mg of sodium chloride (per 100 g of wet cells) were added to the opalescent supernatant and the LPS again precipitated by the addition of 2 volumes of cold acetone. The precipitate was removed by centrifugation, washed with acetone, and dried over sodium hydroxide in a vacuum.

b. Phenol-chloroform-petroleum ether (PCP) method of Galanos et al. for the extraction of *R* type LPSs

Dried bacteria (50 g) were placed in a centrifuge vessel and the extraction mixture containing liquid phenol (90 g dry phenol and 11 mol water), chloroform and petroleum ether in a volume ratio of 2 : 5 : 8, respectively was added (200 ml). The bacteria were then centrifuged (5000 rpm, 15 min) and the supernatant (containing the lipopolysaccharide) was filtered through filter paper into a round flask. The bacterial residue was extracted once more with the same amount of extraction mixture as above and the supernatant was added to the first extract. Petroleum ether and chloroform were then

removed completely on a rotary evaporator at 30 – 40°C. If the remaining phenol would crystallise, sufficient water was added to dissolve it. Water was added drop wise to the solution until the lipopolysaccharide precipitated. Addition of water was stopped when the lipopolysaccharide started settling down after the mixture was allowed to stand for 1 to 2 min. The precipitated lipopolysaccharide was then centrifuged (3000 rpm, 10 min), the supernatant decanted, and the tube allowed to stand for 2 to 3 min upside down. It was then wiped inside with filter paper. The precipitate was washed two to three times with small portions of 80 % phenol (about 5 ml) and the inside of the tube was wiped with filter paper after decantation of the supernatant. Finally, the precipitate was washed three times with ether to remove any remaining phenol, and dried in vacuum. The lipopolysaccharide was taken up in distilled water (50 ml), warmed to about 45°C, and vacuum was carefully applied to remove the air. It was then shaken for a few minutes whereby a viscous solution was obtained. The lipopolysaccharide solution was centrifuged once at high speed (100 000×*g*, 4 h). The resulting sediment was clear and transparent. The lipopolysaccharide was redissolved in water and freeze-dried.

Appendix 2. Silver staining of the slab gels

Step 1. Fix LPS in polyacrylamide gel by placing the gel into a 40 % isopropanol – 5 % acetic acid solution (100 ml) in a clean plastic dish (20 cm square, 5 cm high) overnight.

Step 2. Add 2 ml 0.7 % periodic acid in 40 % ethanol – 5 % acetic acid to oxidize the LPS for 15 min while shaking the dish.

Step 3. Perform three 45-min washes in a second dish with 200 to 500 ml of distilled water per wash.

Step 4. Drain the water off, pour in freshly prepared staining reagent (73.6 ml) and agitate vigorously for 10 min. The staining reagent is prepared as follows. 1 ml of concentrated ammonium hydroxide and 1.4 ml 1 M sodium hydroxide is added to 70 ml water. 1.2 ml of 20 m/v % silver nitrate is then added to the solution while it is being stirred. A transient brown precipitate will form but it will disappear within seconds. The reagent is discarded after use.

Step 5. Perform three 10-min washes as in Step 3.

Step 6. Replace the water with formaldehyde developer (100 ml) containing 2.5 m/v % sodium carbonate and 27 µL formaldehyde. The LPS in the gel will be stained dark

brown within 20 min. Development is terminated with 50 mM EDTA (ethylenediaminetetraacetic acid) when the stain reaches the desired intensity or when the clear gel background shows the first signs of dislocation.

Step 7. Wash and store the gel in water.

Appendix 3. Coating of the inner wall of the quartz electrophoresis capillary with a monomolecular polymer layer to eliminate electroendosmosis and adsorption of solutes

This method was based on a bifunctional compound in which one group reacted specifically with the glass wall and the other with a monomer taking part in a polymerization process. Bind-silane (γ-methacryloxypropyltrimethoxysilane) was used as bifunctional compound and acrylamide as monomer. The methoxy groups in Bind-silane react with the silanol groups in the glass wall, whereas the acryl groups with the acryl monomers to form non-cross-linked polyacrylamide. Non-covalently attached polymer was then removed and a thin, well defined monomolecular layer of polyacrylamide remained covalently bound to the fused silica wall. A detailed description of the whole procedure is given below.

Firstly, a 3 mm detection window was formed on the capillary by burning off the polyimide coating, approximately 8 cm from one end of the capillary (the total length of the capillary was 33 cm). Then in the pre-treatment step the capillary was washed with methanol for 3 min, 1 M NaOH for 3 min, 1 M HCl for 3 min and finally water for 5 min in order to obtain a fresh and clean inner capillary surface, *i.e.*, to improve the homogeneity of the surface structure and thus the reproducibility of the coating and consequently of the runs. Technically, when washing the capillary one end was immersed in the washing solution and the other end connected to vacuum simply by pressing together the vacuum-tube around the capillary. Following drying with an air stream, the capillaries were filled with a 50% solution of Bind-silane in acetone, left for 20 h (while both ends of the capillary were immersed in the same solution) and then rinsed with acetone for 30 s. The polymerization was carried out with 150 µl of de-aerated acrylamide (4 m/v %) containing 3 µl of ammonium persulfate (5 m/v %) as the initiator and 3 µl of TEMED (5 v/v %) as the catalizator. After 90 min, the excess of (not attached) polyacrylamide was removed simply by rinsing with water delivered by an HPLC pump.

Appendix 4. Molecular masses of the constituents of endotoxins

Name	Abbreviation	Molecular formula	Monoisotopic mass (Da)	Average mass (Da)
Fatty acids				
lauric acid	C12	$C_{12}H_{24}O_2$	200.18	200.32
myristic acid	C14	$C_{14}H_{28}O_2$	228.21	228.37
hydroxymyristic acid	C14-OH	$C_{14}H_{28}O_3$	244.20	244.37
unsaturated myristic acid	C14unsat	$C_{14}H_{26}O_2$	226.19	226.36
palmitic acid	C16	$C_{16}H_{32}O_2$	256.24	256.43
Sugar residues				
glucosamine	-	$C_6H_{13}NO_5$	179.08	179.16
D-glucose	Glu	$C_6H_{12}O_6$	180.06	180.16
D-galactose	Gal	$C_6H_{12}O_6$	180.06	180.16
heptose	Hep	$C_7H_{14}O_7$	210.07	210.18
2-keto-3-deoxyoctonic acid	Kdo	$C_8H_{14}O_8$	238.07	238.19
N-acetyl-D-glucosamine	GluN	$C_8H_{15}NO_6$	221.09	221.21
glucuronic acid	GluA	$C_6H_{10}O_7$	194.04	194.14
4-amino-4-deoxy-L-arabinose	L-Ara4N	$C_5H_{11}NO_4$	149.07	149.15
Phosphate	P	H_3PO_4	97.98	98.00
Water	-	H_2O	18.01	18.02

By coupling two constituents the condensation reaction will result in a release of one water molecule, thus, the resulting molecular mass of the sum of *n* molecules will be less with *n-1* water molecular mass unit.

Appendix 5. Interpretations of the main MALDI-TOF MS negative-ion peaks

The first column contains the ions appearing in the spectra in Figs. 25, 28, 30, 32 and 34; the second column indicates the acylation degree of the Lipid A parts in the ions; and the third column indicates the number and type of constituents composing the molecular species in the measeured LPSs or Lipid A-s. Abbreviations used are: C12 for lauric acid, C14 for myristic acid, C14-OH for hydroxymyristic acid, C14unsat for unsaturated myristic acid, C16 for palmitic acid, P for phosphate, Kdo for 2-keto-3-deoxyoctonic acid, Hep for heptose, L-Ara4N for 4-amino-4-deoxy-L-arabinose, Gal for D-galactose and Glu for D-glucose. The “repeating unit of *E. coli* O83 LPS” consists of

one Glu, two Gal, one GluN (N-acetyl-D-glucosamine) and one GluA (glucuronic acid) units.

Observed ion (*m/z*)	**Acyl substitution**	**Fatty acid, phosphate and carbohydrate composition**
1054	tri-acyl	1×C12, 2×C14-OH, 1P
1134	tri-acyl	1×C12, 2×C14-OH, 2P
1158	tri-acyl	1×C14unsat, 2×C14-OH, 2P
1178	tri-acyl	3×C14-OH, 2P
1281	tetra-acyl	1×C12, 3×C14-OH, 1P
1361(63)	tetra-acyl	1×C12, 3×C14-OH, 2P
1489	penta-acyl	1×C12, 3×C14-OH, 1×C14unsat, 1P
1569(70)	penta-acyl	1×C12, 3×C14-OH, 1×C14unsat, 2P
1587	penta-acyl	1×C12, 4×C14-OH, 2P
1597	penta-acyl	1×C12, 3×C14-OH, 1×C16, 2P
1717	hexa-acyl	1×C12, 1×C14, 4×C14-OH, 1P
1769	hexa-acyl	2×C12, 4×C14-OH, 2P
1797(98) refl. m. 1805 lin. m.	hexa-acyl	1×C12, 1×C14, 4×C14-OH, 2P
1826	hexa-acyl	1×C12, 4×C14-OH, 1×C16, 2P
1959	tri-acyl	1134 and 2 Kdo, 2 Hep
2035(36)	hepta-acyl	1×C12, 1×C14, 4×C14-OH, 1×C16, 2P
2087	tri-acyl	1160 and 2 Kdo, 2 Hep, 1P
2166	hepta-acyl	1×C12, 1×C14, 4×C14-OH, 1×C16, 2P, 1× L-Ara4N
2185	tetra-acyl	1160 and 2 Kdo, 2 Hep
2238	hexa-acyl	1×C12, 1×C14, 4×C14-OH, 2P, 2 Kdo
2265	tetra-acyl	1160 and 2 Kdo, 2 Hep, 1P
2369	hepta-acyl	1×C12, 1×C14, 4×C14-OH, 2P, 2 Kdo, 1× L-Ara4N
2394	penta-acyl	1569 and 2 Kdo, 2 Hep
2473	penta-acyl	1569 and 2 Kdo, 2 Hep, 1P
2476	hepta-acyl	1×C12, 1×C14, 4×C14-OH, 1×C16, 2P, 2 Kdo
2607	hepta-acyl	1×C12, 1×C14, 4×C14-OH, 1×C16, 2P, 2 Kdo, 1× L-Ara4N
2622	hexa-acyl	1797 and 2 Kdo, 2 Hep
2703	hexa-acyl	1797 and 2 Kdo, 2 Hep, 1P
3868	hexa-acyl	1×C12, 4×C14-OH, 1×C16, 2P, 3 Kdo, 3 Hep, 3 Gal, 3 Glu
4734	hexa-acyl	3868 and 1×repeating unit of *E. coli* O83 LPS
5600	hexa-acyl	3868 and 2×repeating units of *E. coli* O83 LPS
6465	hexa-acyl	3868 and 3×repeating units of *E. coli* O83 LPS

Observe, that the hexa-acylated Lipid A form measured in reflectron mode (refl. m.) has m/z 1797 (or 1798) values, whereas it appears at m/z 1805 when measured in linear mode (lin. m.).

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