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Structural characteristics of bacterial endotoxins

PhD thesis

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Introduction

The structural composition of bacterial endotoxins (also known as lipopolysaccharides or LPSs) is of importance in their physiological impact. The endotoxins are heat-stable, non-proteinaceous, immunogenic compounds and they cannot be converted to toxoids. They are part of the outer membrane of the Gram-negative bacterial cell wall. Lipopolysaccharides can be divided into three distinct regions: namely lipid A, core and O-specific chain. The **lipid A** part is a hydrophobic structure directed towards the interior of the cell, which is responsible for the toxic biological effect. Regarding its conservative structure, it is usually a bis-phosphorylated disaccharide backbone acylated with fatty acid chains. The **core** is the connection part between the lipid A and the O-specific chain and can be divided into two subunits: the *inner core* and the *outer core*. The inner core has the same composition in all Gram-negative bacteria, containing rarely occuring components (heptose and 3-deoxy-D-manno-2-octulonic acid (Kdo)). The outer core is made of up to six hexose components. The outermost element of the LPS structure is the hydrophilic **O-specific chain**, which is built up from 1-40 repeating units (consisting of 2-7 sugar components) resulting in diverse composition. The repeating units are characteristic for the bacteria, resulting in a large variety of antigenicity, which is the base of the serological grouping of Gram-negative bacteria. The presence or absence of the O-specific chains determines the phenotype of the LPS:

- *R*-type, where the O-specific chain is absent, resulting in *rough* surface of the bacterial colonies. Bacterial strains with *R*-type LPSs are non-pathogenic mutant strains;
- *S*-type, where the O-specific chain is present, resulting in *smooth* surface of the bacterial colonies. Bacterial strains with *S*-type LPSs are pathogenic wild-type strains.

The detection of endotoxins is in the interest of both healthcare and pharmaceutical industry. There are several methods known with various sensitivity, however, since the withdrawal of the LAL-assay in summer 2011, no standard assay exists for endotoxin detection, that could be generally used worldwide.

When it comes to the structural analysis of lipopolysaccharides, whether it is hydrolyzed or not, numerous methods should be mentioned, e.g. gel electrophoresis, TLC, GC, CE, NMR or MALDI. However, none of these techniques is limitation-free, therefore a combination of them should be applied to achieve the most accurate results. In my thesis the results and experiments for the development of a rapid and reliable capillary electrophoretic method for the characterization of endotoxins along with GC-MS and MALDI-TOF methods will be described.

Aims

The main goal of this study was to describe the structural components and the structure of the core part of endotoxins from *S. sonnei R*-type isogenic mutant strains, since these strains are generated from the same *S*-type parent strain (*S. sonnei* phase I) assuming similarities in their structures. To achieve this goal, the detailed aims of this study were the followings:

1. To develop a suitable CE-LIF method, specialized for the detection of various carbohydrate components, by applying the fluorescent dye APTS, in order to apply the developed method in the investigation of bacterial endotoxins

2. To identify the carbohydrate components of the hydrolyzed core part of bacterial endotoxins from *S. sonnei R*-type isogenic mutant strains with the help of the developed CE-LIF method

3. To identify the carbohydrate components of the hydrolyzed core part of bacterial endotoxins from *S. sonnei R*-type isogenic mutant strains by GC-MS measurements

4. To determine the structural homologies and differences of the core part of bacterial endotoxins from *S. sonnei R*-type isogenic mutant strains by MALDI-TOF measurements

5. To describe the various structures of the core part of bacterial endotoxins from a series of the mutant *Shigella sonnei R*-type strains based on various analytical means, in an increasing order of complexity.

Materials and methods

Bacterial endotoxins

The *Shigella sonnei* „*R*" mutant strains (*4350, 562H, R41, 4303*) were isolated and extracted in the Department of Medical Microbiology and Immunology, Faculty of Medicine, University of Pécs. Bacteria were cultured in a laboratory fermentor at 37°C.
After phenol-water extraction and lyophilization, the endotoxins were hydrolyzed with 1 % acetic acid, to separate the carbohydrate part from the lipid A part. The resulting oligosaccharides were purified with column chromatography, and carbohydrate-containing fractions were further hydrolyzed with 0.5 M sulphuric acid to obtain the monosaccharide components of the core part. The hydrolyzed endotoxin-samples were filtered and lyophilized.

GC-MS Examinations

The endotoxin-hydrolyzates were examined by GC-MS in their alditol-acetate derivative form after reduction and peracetylation. Samples containing the sugar derivatives were desiccated and then resuspended in chloroform.
The experiments were carried out on a GC-MS system consisting of an Agilent 6890N gas-chromatograph coupled to an Agilent 5975 mass spectrometer. The separation was performed on an Agilent DB-225 capillary column. The monosaccharides from the hydrolyzed endotoxin-samples were identified with the help of the MS-library and with the addition of known standards to the samples (so-called spiking). Inositol was used as internal standard in its alditol-acetate form, since none of the samples contains it naturally.

CE-LIF

0.1 M mono- and oligosaccharide standards and 2 mg/ml aquaeous solutions from the endotoxin-hydrolyzates were prepared as stock solutions. Samples were derivatized with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) fluorescent dye via reductive amination.
Capillary electrophoretic experiments of the fluorescently labeled samples were performed on a Crystal 300 CE system equipped with a Zetalif laser-induced fluorescence (LIF) detector. Experimental conditions were as follows: 30 kV voltage, about 21 μA current, 120 mM borate pH 10.2 background electrolyte. The identification of the fluorescent derivatives of the

samples was done by the addition of derivatized known standards to the samples (so-called spiking) and based on the GC-MS results.

MALDI-TOF-MS

1 mg of intact LPS samples extracted from *Shigella sonnei 4350, 562H, R41* and *4303* strains were suspended in 0.5 ml of 0.1 M citric acid aqueous solution. Samples were desalted using Dowex 50WX8-200 (H^+) cation-exchange resin. Afterward sample was deposited on a stainless steel target and mixed with 2,5-dihydroxy benzoic acid (DHB) matrix and analyzed immediately after drying.

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry **(**MALDI-TOF MS) measurements were performed on an Autoflex II MALDI-TOF/TOF MS instrument equipped with a 1.2 m drift tube. The spectra were recorded in the negative-ion and linear mode over the 800–4000 *m/z* range. The evaluation of the MS spectra was made considering the following average mass units for the calculation of molecular masses of the ions: GlcN (*β*-1–6-linked GlcN) disaccharide: 340.33; C14(3-OH) (3-hydroxytetradecanoic acid): 226.36; C14 (tetradecanoic acid): 210.36; C14unsat (unsaturated tetradecanoic acid): 208.34; C12 (dodecanoic acid): 182.31; Kdo (3-deoxy-D-manno-2-octulosonic acid): 220.18; Hep (heptose): 192.17; Hex (hexose): 162.14; P (phosphate): 79.98; H: 1.01.

Results and discussion

Development of the CE-LIF method for the examination of carbohydrate components

To develop a sensitive method for carbohydrate detection, the methodological problems in the labeling efficiency of mono- and oligosaccharides were studied. The conjugation of sugars was modelled by labeling with APTS separately and in mixtures. The efficiency of the separation and the labeling was followed by CZE of dye-conjugate mixtures. It was shown that both, the qualitative and quantitative determination of the sugars in mixtures need a careful optimization.

To verify the separation efficiency, fifteen mono- and oligosaccharides were labeled separately with APTS (at 1:1 dye:carbohydrate molar ratio). After labeling, the conjugates were mixed (1:1 molar ratios of the initial concentrations of the different carbohydrate substances) and submitted to electrophoresis. The labeled monosaccharides migrated separately and appeared with different peak areas in the electropherograms, except the APTS

conjugates of glucose, ribose and saccharose were co-migrating. We could also observe that conjugates of fructose, saccharose, stachyose and the amino-sugars (galactosamine and glucosamine) were always appearing as relatively small peaks in the electropherograms. We suggest, that aminosugars are labeled with a lower yield because of the differences in the basicity of the amino-groups. To avoid this phenomenon, acetylation of the aminosugars should be performed or other, amino-group targeting derivatizing agents may be applied. The lower labeling yield of stachyose is probably due to its larger size. Also, the relatively low yield of fructose-APTS conjugates is probably due to its structural difference compared to the other hexose monosaccharides.

In order to examine the efficacy of labeling and the potentially occuring competitive effect, sugar molecules were labeled separately and in mixture with various molar ratios of the sugars to the dye. In the case of sugars labeled separately, changing the molar ratio from 1:7 up to 4:1 (APTS:carbohydrate, respectively) no changes in the labeling behaviour of the components were observed. When, however, mixtures of monosaccharides were labeled with APTS, the relative peak areas represented uneven labeling of the different molecules. Applying an APTS to "total carbohydrate" ratio 1:7 or 1:1, respectively, some sugars were labeled with less efficiency, or not labeled at all. For example the aminosugars appeared only in the electropherograms if the ratio was at least 1:1, but the same peak area ratios were observed only if the APTS:total carbohydrate ratio was at least (or higher than) 2:1. When carbohydrate-mixtures are labelled, the competitive labeling of the components might result different ratios. This competitive effect can be overcome with the use of dye-excess.

Carbohydrate constituents and structures of the *R*-type endotoxins

To identify the carbohydrate constituents of the core part of the endotoxins, Lipid A was removed after mild acid hydrolysis of the intact LPS samples from *S. sonnei* isogenic rough mutants, and the truncated core parts of the different LPSs were further hydrolyzed to obtain monosaccharide residues. The derivatives of monosaccharide residues were then analyzed by GC-MS and CE-LIF.

The intact LPSs – extracted from the isogenic *Shigella sonnei R*-mutants – were examined with MALDI-TOF-MS. The MS spectra exhibit complex pattern indicating heterogeneities in both, the lipid A parts and the core OS parts.

In all spectra, the quasimolecular ions $[M–H]^-$ at *m/z* 1134, 1361, 1569 and 1797 were observed corresponding to bis-phosphorylated tri-, tetra-, penta- and hexa-acylated lipid A species, respectively.

In the case of *S. sonnei 4350* no monosaccharide residues were found in the hydrolyzed fraction by either of the separation methods. In the MALDI-TOF experiments, additionally to the ions corresponding to the differently acylated lipid A species, major ions were observed at *m/z* 1574.3, 1800.2, 2008.9 and 2237.5 corresponding to the respective lipid A species carrying two Kdo molecules. Based on our results, *S. sonnei 4350* – previously reported as a heptose-transferase-less mutant – is an absolute rough mutant having only two Kdo molecules in its core region.

The GC-MS and CE-LIF analyses of the hydrolyzed core samples from *S. sonnei 562H* LPS showed the presence of only one type of monosaccharide components. Although we did not have heptose standard for spiking, but we could rely on the mass spectrometric data from GC-MS experiments, we could identify the peak as a heptose-derivative, presumably D-glycero-D-mannoheptose, with a relative retention time of 1.44 in the GC-MS experiments. In the MALDI-TOF spectrum of intact LPSs tri-, tetra-, penta- and hexa-acylated LPS species containing two Kdo molecules were observed at *m/z* values 1556.6, 1575.0, 1783.0, 1801.1, 2010.4 and 2237.4, respectively. Peaks at *m/z* 1766.9, 1993.1, 2219.8 and 2429.4 were identified as the LPS quasimolecular ions containing the tri-, tetra-, penta- or hexa-acylated lipid A moiety and two Kdo and one heptose units. *S. sonnei 562H* LPS has two Kdo residues and one DD-heptose residue in the core, confirming previous studies, which revealed the lack of the ADP-L-glycero-D-mannoheptose 6-epimerase enzyme.

The GC-MS and CE-LIF analyses of the hydrolyzed core samples from *R41* LPS showed the presence of only heptose monosaccharide components. Although we did not have heptose standard for spiking, but we could rely on the mass spectrometric data from GC-MS experiments, we could identify the peak as a heptose-derivative, with a relative retention time of 1.59 in the GC-MS experiments. This result suggests, that the heptose in *S. sonnei 562H* and the one in *R41* somehow differs from each other, presuming L-glycero-D-mannoheptose as the constituent of the *S. sonnei R41* endotoxin core. The intact LPSs were examined by MALDI-TOF also in the case of *S. sonnei R41*. In these experiments, besides the peaks corresponding to the tri-, tetra-, penta- and hexa-acylated lipid A species, another series of peaks could be detected, where *m/z* 1959.7, 2185.3, 2412.1 and 2622.4 values correspond to the intact LPS moieties containing the core OS linked to the differently acylated lipid A molecules. The *ca*. 824 Da differences indicate a core structure containing two Kdo and two heptose units.

The monosaccharide constituents of *R*-type endotoxin from *S. sonnei 4303* mutant was also examined and three monosaccharides were detected by GC-MS and CE-LIF. In the GC-MS experiments, three peaks could be observed, corresponding to galactose, glucose and heptose constituents, where the estimated molar ratio of galactose and glucose was found to be 2.0:3.1, and the relative retention times of the alditol acetate derivatives of galactose, glucose and heptose were 0.91, 0.96 and 1.60, respectively. Accordingly, this mutant contains the same type heptose as the *S. sonnei R41* mutant strain, *i.e.* L-glycero-D-mannoheptose. During the CE-LIF analysis, the fluorescent derivatives of the heptose and glucose constituents in the *4303* LPS sample comigrated, but separated from the fluorescent derivative of galactose. Hexoses were identified with spiking the sample with derivatized known standards, while due to the lack of a heptose standard, heptose was identified based on the GC-MS results.
Concerning the intact LPSs from *S. sonnei 4303*, three regions could be identified in the MALDI-TOF-MS spectrum, signals corresponding to (i) the intact *R*-type LPS structures, and to (ii) lipid A species (as Y-type fragment ions) between *m/z* 3000 – 4000 and to (iii) OS moieties (as B-type fragment ions) between *m/z* 1000 – 2100. The ions at *m/z* 1134.5, 1360.9, 1568.7 and 1798.0 – also observed in the case of the other isogenic mutant strains - correspond to bis-phosphorylated tri-, tetra-, penta- and hexa-acylated lipid A species, respectively. The mass differences (*ca*. 1988 Da) indicate a core structure consisting of two Kdo, three heptose, five hexose, and two phosphate groups. In the *m/z* 1000 – 2100 region, the signals corresponding to OS species of the core (B-type ions) were also observed.

Since we have encountered differences concerning the heptose constituents during GC-MS experiments and in the CE-LIF experiments results were not evidently supporting, the different samples were mixed together to verify, whether we deal really with two different heptoses or not. Results were reassuringly clear, since both in GC-MS and CE-LIF experiments the mixture of endotoxin hydrolyzates from *S. sonnei 562H* and *4303* resulted in five (with the peak of the IS) or three peaks, respectively. Since we already knew, that the derivative of the heptose of *S. sonnei 4303* migrates together with the derivative of the glucose in CE-LIF experiments, it was now obvious, that the heptoses are different from each other. In the case of CE-LIF experiments, identification of the peaks was performed as before; using APTS-derivatized standards for the hexoses and rely on the GC-MS data concerning the heptoses. In the GC-MS and CE-LIF experiments, the two examined saccharide derivatives were fully separable from each other, leaving no doubts about the differences in the heptose content of the endotoxins from different *S. sonnei* mutant strains.

The proposed chemical composition and structural features of the LPSs of the four mutants (*S. sonnei 4350, 562H, R41*and *4303*), are summarized in **Figure 1.**

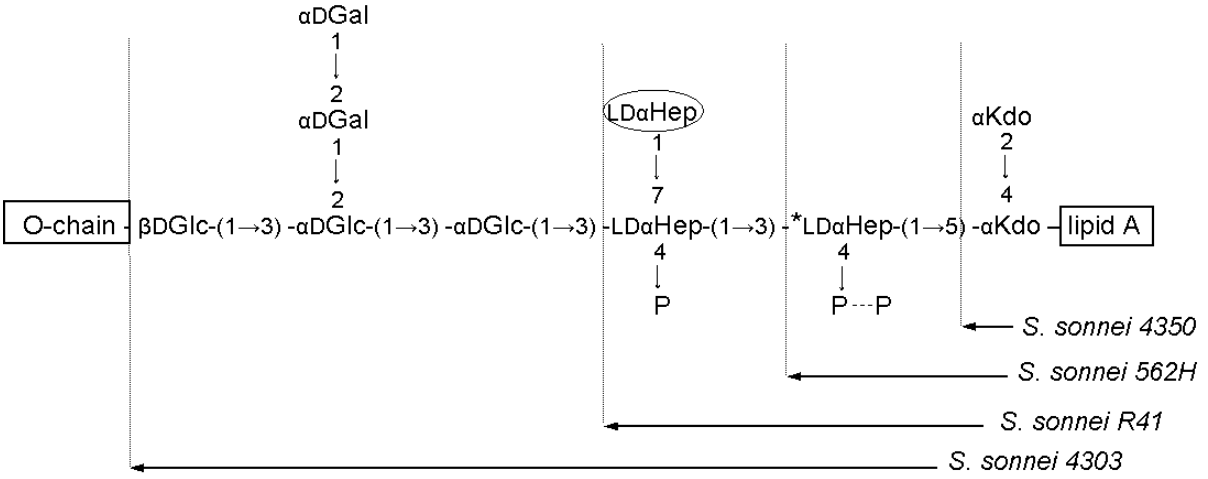

Figure 1. The chemical structures of the R-type endotoxins from the isogenic *Shigella sonnei* mutants, according to the present study and previous investigations. The *S. sonnei 562H* mutant LPS contains D-glycero-D-mannoheptose (*), while *S. sonnei R41* and *4303* LPS contain two or three L-glycero-D-mannoheptoses, respectively (the LD-heptose in the circle is a constituent only in the endotoxin from *S. sonnei 4303*). The "O-chain" gives a hint to the S-type *S. sonnei* phase I endotoxin structure. Kdo: 3-deoxy-D-manno-2-octulosonic acid, Hep: heptose, Glc: D–glucose, Gal: D–galactose.

Conclusions

The experimental circumstances for labeling carbohydrates with 8-aminopyrene-1,3,6-trisulfonic acid were studied and the labeling efficiency of mono and oligosaccharides present in endotoxins was followed by capillary electrophoresis using LIF detection.

The structural variations in the rough-type endotoxins of *Shigella sonnei* mutant strains were investigated by CE-LIF, GC-MS and MALDI-TOF-MS. It was found that the LPSs of the *S. sonnei* isogenic rough mutants *4350, 562H, R41* and *4303* were formed in a step-like manner. This study provides a comprehensive comparison of the variability in rough endotoxin extracted from *S. sonnei* mutants. We can conclude that the studies to identify and quantify the components of oligo- and polysaccharides need a careful experimental approach.

Theses of the work:

1. an effective CE-LIF method was developed for the examination of APTS-labeled carbohydrate mixtures of unknown composition. The methodological problems were studied and labeling efficiency was optimized for carbohydrate mixtures
2. the carbohydrate components of the core part from isogenic rough mutant *S. sonnei 4350, 562H, R41* and *4303* bacterial strains were separated and identified with the help of the developed CE-LIF method, after hydrolyzation and APTS-derivatization
3. the carbohydrate components of the core part from isogenic rough mutant *S. sonnei 4350, 562H, R41* and *4303* bacterial strains were separated, identified and quantitated with the help of by GC-MS, after hydrolyzation and alditol acetate derivatization
4. the structure of the core part and the lipid A moiety has been determined for the LPSs of the above mentioned bacterial strains with the help of MALDI-TOF MS. Molecular masses of intact LPSs were determined
5. the full composition and structure of intact LPSs from *S. sonnei 4350, 562H, R41* and *4303* were described by combining the results of MALDI-TOF experiments with those achieved from GC-MS and CE-LIF experiments.

It is important to know the exact composition of the endotoxin from a certain pathogenic bacterial strain, since serological cross-reactions can be misleading. Our novel capillary electrophoretic method is a fast and very sensitive method for the detection of carbohydrate mixtures obtained from bacterial LPS. Although, this work concerns only *R*-type bacterial strains, it is possible, in the future, to apply this method together with mass spectrometric experiments, to not only *R*-, but also to *S*-type endotoxins, allowing us to determine the fine structure and composition of the O-specific polysaccharide chain from the endotoxin of pathogenic species.

Publications related to the thesis

Bui, A., Kocsis, B., and Kilár, F. (2008), Methodology to label mixed carbohydrate components by APTS, *Journal of Biochemical and Biophysical Methods,* **70 (6)**, 1313-1316.
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Bui, A., Kilár, A., Dörnyei, Á.,Poór, V., Kovács, K., Kocsis, B., Kilár, F. (2011), Carbohydrate composition of endotoxins from R-type isogenic mutants of Shigella sonnei studied by capillary electrophoresis and GC-MS, *Croatica Chemica Acta* ,**84 (3)**, 393-398.
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Kilár, F., **Bui, A.**, Kilár, A., Kocsis, B., Szabó, Z., Farkas, V.: Study of structure-function relationship in endotoxin analysis by microchips and mass spectrometry
22nd International Symposium on Microscale Bioseparations, MSB2008
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