UNIVERSITY OF PÉCS

Doctoral School of Chemistry

Characterization of bacterial endotoxins by chip techniques, structure and immune function relationship

PhD Thesis

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1 Introduction

The presence of microbial pathogens in the bloodstream triggers systemic inflammation and can lead to sepsis, which often defeats the most powerful antibiotic therapies and causes multiorgan systems failure, septic shock and death. Sepsis afflicts 18 million people worldwide every year, with a 30–50% mortality rate even in state-of-the-art hospital intensive care units, and its incidence is increasing because of the emergence of antibiotic-resistant microorganisms. The main cause of sepsis is the immune response to the pathogens (bacteria, viruses, fungi and other microbes and their metabolites) in the blood.

The number of specific pathogen suddenly increases, and the immune system "overreact" to the infection, which leads to coagulation, vascular congestion, edema, systemic inflammation of the tissue, and eventually septic shock under astonishingly short time. Significant amounts of endotoxins were liberated from disintegration of bacteria by antibiotics treatment which starts up some type of chain reactions. Sepsis treatment usually involves the use of empiric, broad-spectrum antibiotic therapy because it takes days to identify the source of the infection and blood cultures are often negative. But these broad-spectrum agents are not as effective as therapeutics targeted against specific microbes, and they can produce severe side effects; as a result, mortality rates increase up to 9% for every hour before the correct antibiotic therapy is administered. This situation is even more devastating in patients with antibiotic-resistant pathogens because of the lack of effective drugs, and in immunosuppressed patients and neonates with fungal infections because of the high toxicity of antifungal agents. The bacterial infection and the prevention of endotoxin contamination within it, is not only medical, but also of commercial interest too.

It is important requirement that, injections, surgical instruments, infusions, dialysis tubes are all sterile and endotoxin-free. The producing companies have to make sure that all their products are free of endotoxin contamination. That’s way the determination of endotoxin is commercially became significant. The detection and quantitative determination of endotoxin contamination is a critical parameter in pharmaceutical and biotechnological processes as well as health services (*e.g.* haemodialysis set)

Our work deals with the endotoxin’s structures and functions, which provides an opportunity in the diagnosis and detection of biological mechanisms.

2 Aims of the theses

To describe the relationship between the structure and function of endotoxins as the following objectives.

1. To develop a microchip electrophoretic method in the structural characterization of various lipopolysaccharide molecules extracted from *Enterobacteriaceae* (*E. coli*, *Salmonella*, *Proteus* genera and *Shigella sonnei* mutants) and to determine the quantitation and molecular masses of endotoxins. To separate heterogeneous S-endotoxins extracted from Gram-negative bacteria by molecular weight, the quantitation of the components of endotoxin molecule mixture and to create the structural type classification. Utilization of electrophoresis and mass spectrometry.

2. To use the improved microchip electrophoretic technique in the characterization of endotoxins (*E. coli*, *Salmonella* and *Proteus* genus) prepared directly from whole-cell lysates, as well as to detect degraded polysaccharides with high sensitivity and rapidity.

3. Taxonomically distant, but serological cross-reacting bacterial endotoxins (*P. morganii* O34 (8662/64), *E. coli* O111 and *S. enterica sv. Adelaide* O35) structural analysis and serological cross-reaction background of mass spectrometry and immunoassay containing colitose.

4. To develop the capillary zone electrophoresis method for biomolecules / endotoxins detection and separation on microchip.

5. To analyze the effect of endotoxins in human red blood cell membrane by microchip electrophoresis method and EPR spin-labeling.

3 Materials and Methods

3.1 Bacterial strains

The bacterial strains used for our experiments were the following: *E. coli* O21, O55, O83, O111, O112, O157, ATCC 25922, D31, *S. enterica sv. Adelaide* O35, *S. enterica sv. Minnesota* wild type, R595, *S. enterica sv. Urbana* O30, *P. morganii* O34, 352, 1594, *P. penneri* 101, 102, 103, 104, 105, *Shigella sonnei* 4303 (phase II), 41, and *Shigella flexneri* 5.

3.2 Endotoxins, lipid A, O-antigens

LPS was extracted (purified) from smooth-type bacteria by **hot phenol/water (1:1/v:v) method** according to *Westphal et al.* and from rough-type strains by **phenol-chloroform petrol ether (2:5.8/v:v:v) method** of *Galanos et al*. and lyophilized. The lipid A and O-antigen components of endotoxins were prepared according to *Kumada et al.* method.

3.3 Gel permeation chromatography

The LPSs were hydrolyzed with 1% (v/v) acetic acid at 100°C for 90 minutes to split the glycosidic linkage between the lipid A part and the polysaccharide part. Then the solution was centrifuged at 8000 x g for 20 min at 4°C. The sediment containing lipid A was washed four times with distilled water and lyophilized. The supernatant was fractionated by gel permeation chromatography on a Sephadex G-50 column (2.5x80 cm) (Sigma Chemicals, St. Louis, MO, USA) using pyridine-acetic acid buffer (4 ml pyridine and 10 ml *cc***.** acetic acid in 1000 ml water) to obtain degraded polysaccharide sample (DPS).

3.4 Partially purified endotoxins samples from whole-cell lysates

Partially purified endotoxin samples were obtained from whole-cell lysates by an enzymatic process. Bacterial strains (from one spot of bacterial colony grown on agar plate) were cultivated in 1 ml medium, heated at 100^{o}C, lysed with lysozyme to destroy the peptidoglycan and LPS lysing buffer, and then subjected to proteolytic digestion with proteinase K enzyme to eliminate the protein components of the Gram-negative bacterial cell wall. After stopping the digestion with magnesium-chloride, the mixture was stored at −20^{o}C, overnight, and the digest was centrifuged (13 000 × g, 15 min). The sediment (containing LPS) was solubilized in 30 µl deionized water and sonicated. These protein-free LPS solutions were analyzed without further purification.

3.5 Microchip gel electrophoresis

Electrophoresis in microchips was performed in the commercially available Agilent 2100 Bioanalyzer system (Agilent Technologies) equipped with a diode laser for fluorescence detection (with 630 and 650 nm as excitation and emission wavelengths, respectively). Separation of 11 endotoxin samples could be achieved in approximately 30 min using a disposable ten-sample separation glass microchip.

The method is a modification of the *High Sensitivity Protein 250* protocol used originally for protein analysis. In details; the LPS solutions (solution of purified LPS in physiologic saline or partially purified LPS from whole-cell lysate) were diluted ten times with Standard Labeling Buffer. For the fluorescent labeling 0.5 µl fluorescent dye/DMSO solution was added to 5 µl diluted sample and incubated for 10 min at room temperature. The fluorescent dye is bound through a nitrogen in the lipid A and/or core part of the LPSs with a molar ratio of 1:1. The excess dye (*i.e.* the unbound dye) in the solutions of labeled LPSs was quenched by adding 0.5 µl ethanolamine following the reaction time. The ethanolamine addition caused the appearance of a fast-migrating system peak (fluorescent dye bound to ethanolamine) in the electrophoretic profile. The labeled samples were diluted five times by adding 24 µl deionized water. Four microliters of this diluted sample solutions were combined with 2 µl "disaggregating solution", incubated at 100^{o}C for 5 min and centrifuged.

The microchip channels were filled hydrodynamically (with pressure) with 12 µl *Gel Matrix* (polydimethylacrylamide-based linear polymer), the sample wells were loaded with samples (6 µl each, containing the covalently labeled LPSs) and the respective wells were loaded with the *Destaining Solution*.

The script of the Bioanalyzer 2100 (defining the operational steps of the microchip analysis) was modified, the injection was made with 1000 V for 80 s (which resulted in an injection volume of *ca.* 40 pl), and the separation was continued toward the anode at 1000 V for 60 s or 90 s at 30^{o}C. The running times were *ca.* 80 s. The raw data were either plotted by the built-in 2100 Expert Software or exported to Excel for presentation. Endotoxin samples were analyzed at least for three times in the same chip and/or in different chips in order to control repeatability and reproducibility of peak positions, peak numbers, and peak areas.

3.6 ELISA tests

ELISA tests were used to determine the immunological cross-reactivity. Briefly, intact LPS, degraded polysaccharides and the lipid A from the *P. morganii* O34, *E. coli* O111 and *S. enterica sv. Adelaide* O35 bacteria were conjugated in microplates and then incubated with rabbit antisera produced against each bacterium. The cross-reaction was followed by the conversion of o-phenylenediamine by peroxidase conjugated goat anti-rabbit-IgG measuring the optical density by a plate reader (Titertek Uniscan reader, Flow Laboratories, Helsinki, Finland) at 492 nm. The "antibody depletion assay" was performed with each antiserum, which was incubated for one night with each type of the heat-killed bacteria at 4°C. The

bacterial cells with the trapped antibodies were sedimented by centrifugation (6000 x g, 20 min) and the supernatant was used as depleted antiserum sample.

3.7 Gas chromatography and mass-spectrometry (GC-MS)

The sugar composition of LPS was analyzed by alditol acetate method according to the *Sawardeker and Sloneker*. Briefly, LPSs were hydrolyzed with 1 M sulfuric acid at 100°C for 14 h. The hydrolysates were neutralized by barium hydroxide and reduced with sodium-borohydride at room temperature for 14 h. The excess borohydride was quenched with acetic acid and then the borate was removed with methanol. The samples containing the reduced sugars were then desiccated. Peracetylation was performed with acetic anhydride at 100°C for 14 h. The samples, containing the sugar derivatives, were washed once with 3 ml distilled water, desiccated and diluted in 1 ml chloroform.

The GC-MS system (Agilent technologies, Waldbronn, Germany) consisted of a gas chromatograph (6890N) and a mass spectrometer (5975) equipped with a quadrupole mass analyzer. The separation was performed on a DB-225 capillary column (Agilent Technologies, Waldbronn, Germany, length: 30 m, inner diameter: 0.25 mm, film thickness: 0.15 μm). Helium was used as a carrier gas (initial flow, 1.5 ml/min) and split injection was applied. The injection volume was 1 μl. The GC temperature program was as follows: the temperature was raised from the initial 180°C to 235°C with a rate of 5°C/min and maintained for 41.50 min. The injector temperature was 200°C, the transfer line was set at 300°C and the ion source was used at 230°C. The mass spectrometer was operated at 70 eV in the electron impact (EI) mode and the scanned mass range was 20-600 amu.

The monosaccharides of the samples from the different bacterial strains were identified with the help of the MS-library and by spiking the samples with standards. Inositol in its alditol-acetate form was used as an internal standard.

3.8 Endotoxin interaction detection by microchip gel electrophoresis and EPR studies

The endotoxin interaction with hemoglobin was analyzed by microchip gel electrophoresis method. 1 mg/ml hemoglobin was treated for 20 min with 2 mg/ml purified endotoxin from *E. coli* O83 in ratio 1:1.

The endotoxin interaction with human red blood cell was analyzed by EPR studies. One ml human whole blood was incubated at 4°C for 90 min with the endotoxin from *E. coli* O83 and *S. enterica sv. Minnesota* R595 (200 μg/ml, 100 μg/ml and 10 μg/ml) then the plasma was removed. Spin-labeling was made with 5-doxylstearic acid (5-SASL, 1.5 mg/ml). EPR spectra

were recorded with an ESP 300E spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a diTC 2007 temperature regulator. The conventional EPR spectra were obtained at a microwave power of 20 mW and a field modulation of 100 kHz, with modulation amplitude of 2.0 G.

The order and motion of the membrane lipids were assessed by measuring the half-width at half-height (HWHH) of the low-field resonance line of the 5-SASL spectrum. A decrease in HWHH indicates a decrease in fluidity.

4 Results and discussion

4.1 Development of microchip gel electrophoresis method for structural characterization of lipopolysaccharides

Endotoxins extracted from different strains of the *Enterobacteriaceae* family were analyzed with MGE using molecular sieving environment. The method originally developed for the analysis of proteins with covalently bound fluorescent dye was modified and applied for the analysis of *S*- and *R*-type endotoxins. The experimental conditions were optimized for the quantitative analyses of endotoxin components from purified LPS or partially purified samples from whole-cell lysates. The following parameters were optimized: ***(i)*** the quantitation of fluorescent dye was decreased (the high-intensity system peak from the ethanolamine + fluorescent dye complex not overlap the rapid mobility lipid + core fluorescent signal intensity that appears in the electropherograms) ***(ii)*** for the faster sample preparation the endotoxins labeling with fluorescent dye was shortened from 30 minutes to 10 minutes (the endotoxin components resulting no change in the intensity of the signal), ***(iii)*** optimized the fluorescent dye-endotoxin complexes' concentrations in the case of the best resolution ***(iv)*** changed the detection time from 60 s to 90 s.

4.1.1 Classification according to type of structure

Endotoxins extracted from different strains of the *Enterobacteriaceae* family were analyzed with MGE using molecular sieving environment. The electrophoretic profiles of the labeled endotoxins showed characteristic distribution of the components after the system peak (the fluorescent dye bound to ethanolamine) for the *S*-type and *R*-type endotoxins (with numerous components or only one or two components, respectively) could be observed.

The electrophoretic separation profiles of fluorescently labeled endotoxin components from the *S*-type strains of the *E. coli*, *Shigella*, *Salmonella*, and *Proteus* genera are analyzed. The

peaks in the electropherogram correspond to LPS components with increasing numbers of repeating units in their O-polysaccharide chain, (*i.e.* the first peak corresponds to a component built from the lipid A and core parts). The *S*-type endotoxins can be characterized by the migration time-range and relative amounts of the components. The endotoxin molecules of *E. coli* O83 and O112 bacteria migrated within less than 38 s, which is a relatively fast migration compared to the other LPSs, and a minimum and maximum in the relative amounts were observed (*group 1a*). Similar profiles with a minimum and maximum in the relative amounts, but longer migration times (peaks up to 58 s) were found for the endotoxin components from *E. coli* O21, O55, O111, O157, 25922, *P. penneri* 101, 102, 103, 104, and *S. flexneri* 5 (*group 1b*). The (relative) amount of the first component compared to the second component was significantly lower in the profiles of LPSs from *P. penneri* 105, *S. enterica sv. Minnesota* wildtype, *S. enterica sv. Adelaide* O35 and *S. enterica sv. Urbana* O30, and moreover molecular components, with a maximum in the peak intensity profile appeared at long migration times (up to 70–75 s) (*group 1c*). The endotoxins extracted from the *P. morganii* O34, 352, and 1594 bacteria include components with gradually decreasing relative amounts in the whole electropherogram (*group 2*).

The electrophoretic profiles of purified *R*-type endotoxin samples extracted from *S. enterica sv. Minnesota* R595, *S. sonnei* R41, 4303 (phase II), and *E. coli* D31 strains. One or two components can be observed, and in several cases, the components overlap with the system peak, which reflects the low molecular masses of these components.

The limit of detection was determined by decreasing the amount of the purified endotoxins in the experiments. For the single LPS component of the rough-type *S. enterica sv. Minnesota* R595, the limit of detection was 1.13 ng/µl. The detection limit of the endotoxin from the *S*-type *E. coli* O83 was 0.43 ng/µl determined considering the peak of the first component in the electropherogram.

4.1.2 Bacterial lysates

Protein-free LPS samples obtained from bacterial lysates were directly analyzed (without further purification) with the new MGE method. The electrophoretic profile of the partially purified LPS in *E. coli* O83 whole-cell lysate is analogue to the purified *S*-chemotype *E. coli* O83 endotoxin. The “wave-like” pattern of 20 peaks is consistent with the profile, although, the relative peak area ratios are slightly different in the two patterns. The analysis of an *R*-type partially purified endotoxin sample prepared from the whole-cell lysate of *S. enterica sv. Minnesota* R595 bacteria and its electropherogram show a single-component profile analogue

to the purified *R*-chemotype endotoxin. The chip LIF-CE afforded high-resolution separation for partially purified (whole-cell lysate) S and R LPSs, as well. Therefore, in combination with the proteinase K enzymatic digestion method, this technique provides unparalleled sensitivity and enables the characterization of LPS surface antigens from as little as one bacterial colony (culture spot on agar-plate).

4.1.3 Determination of the molecular masses of the LPS components

The molecular masses of LPS components with known structures can be calculated. A number of LPSs have been investigated with MS or NMR. The mass data of LPS constituents, *i.e.* lipid A parts, core parts, and repeating units, are summarize. For the endotoxins, of which all three data are known, it is possible to construct the *logM versus 1/t* diagrams. We found that two calibration lines could be constructed for the known endotoxin components. Molecules belonging to *group 1a* have a different *logM-1/t* line than the others. Applying these calibration curves for endotoxin preparations, of which the molecular structure (masses) of one or more constituents are not known, we could estimate the molecular masses of the components. The calculated mass data for partial structures of endotoxins, *i.e.* lipid A + core and/or the repeating unit, for which no structural data have been reported so far. The error for the calculation was estimated from a calculation of the mass data using the electropherogram for an endotoxin with a known structure.

4.1.4 Detection of degraded O-polysaccharides by MGE method

The fractions of the hydrolyzed *E. coli* O83 LPS were separated on Sephadex column and they were run on the microchip. The resulted electropherograms showed that the 1. fraction corresponds to the total, unhydrolyzed LPS, 2. and 3. fractions correspond to the DPS oligosaccharides (core + degraded, different numbers of repeating units in the O-side chain) and 5.fraction contained only monosaccharides (core).

4.2 Serological cross-reactions and study of their structural background

4.2.1 ELISA tests

Two kinds of ELISA tests were performed to characterize further the cross-reactivity of the bacterial endotoxins. The ELISA results obtained for the immunological reaction between the intact LPS, or DPS or lipid A samples from the bacteria and the antisera produced in rabbits. The cross-binding capacity was followed by measuring the optical densities at 492 nm after

the conversion of o-phenylene-diamine by peroxidase conjugated with anti-rabbit IgG produced in goat. Strong cross-reaction was observed in the cases of the *E. coli* O111 and *S. enterica sv. Adelaide* O35 LPS samples (the OD_{492} fell in the range of 1.15-1.36), while slightly lower interaction was observed for the *P. morganii* O34 LPS was (OD_{492} was between 0.87-1.09). The degraded polysaccharides and the lipid A samples from all three bacteria showed cross-reactions similar to each-other with any of the cross-reacting sera, but with significantly lower intensity compared to the intact lipopolysaccharides.

The results of the ELISA "antibody depletion assay" obtained with antisera depleted with heat-killed cells of the bacteria. All antisera which were treated with either heat-killed bacterium showed extremely diminished ELISA reaction compared to the untreated rabbit antisera produced against the different bacteria. This proves that the serological similarity is very high between the three strains.

4.2.2 GC-MS studies of O-polysaccharide

Gas chromatograms of the alditol-acetate derivatives of the hydrolyzed O-polysaccharides showed similarity in the monosaccharide contents. D-glucose, D-galactose, glucosamine and colitose were detected in the hydrolytic mixtures of each LPS. The identification of the carbohydrates in the evaluation of the GC-MS experiments was accomplished by the help of the MS spectra of the sugar derivatives, and comparison of the relative retention times (inositol was used as internal standard). The relative retention times were also necessary for the assignment of the sugar units with different configuration, but equal molecular masses (*e.g.*, to differentiate between galactose and glucose). The molar ratio of the D-glucose, D-galactose and the glucosamine monosaccharides content were found to be 1:1:1.

4.3 Investigation of the endotoxins' effects on erythrocyte membrane

The effect of the endotoxin on the erythrocyte was studied by EPR method. A significant concentration-dependent decrease in fluidity was induced by the LPSs from *E. coli* O83, reflected by a decrease in the HWHH (*half-width at half-height*) (by ordering the plasma membrane of the treated red blood cell).

The Gram-negative sepsis and endotoxemia are known to be associated with alteration in the red blood cell membrane, which results in diminished flexibility detected by EPR measurements. The sequence of this alteration is thought to be the inability of the red blood cell to alter its shape to travel capillaries which results in a reduction of blood flow through

the microcirculation, decreasing oxygen delivery to the tissues and leading at last to organ damage.

The endotoxin extracted from *E. coli* O83 bacteria interacts with hemoglobin was detected by microchip gel electrophoresis method. The shift in the migration of the hemoglobin subunits and its oligomers shows that a nonspecific hydrophobic interaction and/or intercalation of lipopolysaccharide components occur in the lipid bilayer.

5 Theses of the work

1. Our newly developed microchip electrophoretic method made the structural characterization of lipopolysaccharide molecules from several enterobacteria (*E. coli*, *Salmonella*, *Proteus* genera and *Shigella sonnei* mutants) and able to quantitative evaluation of components of an endotoxin extract. The developed microchip gel electrophoresis and microchip zone electrophoresis method is suitable for the determination of endotoxins labeled with covalent binding of a fluorescent dye.

2. The classification of the LPSs can be done according to their electrophoretic profiles, which are characteristic of the respective bacterial strains. According to the number, distribution, and the relative amounts of an endotoxin extract, we could differentiate the S-type endotoxins.

3. The MGE afforded high-resolution separation of partially purified (whole-cell lysate) S and R endotoxins, and degraded polysaccharides. This microchip technique provides a new, standardisable, fast, and sensitive method for the detection of endotoxins and for the quantitative evaluation of components of an endotoxin extract.

4. Serological cross-reaction background was detected with taxonomically distant bacterial strains (*P. morganii* O34 (8662/64), *E. coli* O111 and *S. enterica sv. Adelaide* O35) by ELISA tests, GC-MS and NMR methods. The GC-MS measurements revealed that all of the O-side chain from those lipopolysaccharides is built from the same type of sugars. Probably the rare sugar, colitose, plays an important role in the cross-reacting ability of the strains examined.

5. We studied the biological effects of endotoxin. Analyze the effect of endotoxins in human red blood cell membrane. Endotoxins reduce the fluidity of human erythrocyte membrane, which follow by EPR spin-labeling. Demonstrated interaction between endotoxin and hemoglobin subunits by microchip gel electrophoresis method.

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7 Publications

Publications related to this thesis

1. Béla Kocsis, Anikó Kilár, **Lilla Makszin**, Krisztina Kovács, Ferenc Kilár: Capillary electrophoresis chips for fingerprinting endotoxin chemotypes from whole-cell lysates, *Microbial Toxins: Methods and Protocols*, 89-99, 2011
2. **Lilla Makszin**, Anikó Kilár, Péter Felső, Zoltán Péterfi, Béla Kocsis, Ferenc Kilár: Quantitative microfluidic analysis of S- and R-type endotoxin components with chip capillary electrophoresis, *Electrophoresis* 2012, 33, 3351–3360. **IF.: 3,261**
3. **Lilla Makszin**, Zoltán Péterfi, Ágnes Blaskó, Viktor Sándor, Anikó Kilár, Ágnes Dörnyei, Erzsébet Ősz, Ferenc Kilár, Béla Kocsis: Structural background for serological cross-reactivity between bacteria of different enterobacterial serotypes, *Electrophoresis* 2015, 36, 1336-1343. **IF: 3,161***

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1. **Makszin Lilla**, Kilár Anikó, Kocsis Béla, Kilár Ferenc: Bakteriális endotoxinok gyors és érzékeny microchip elektroforetikus kimutatása, Elválasztástudományi Vándorgyűlés, 2008. november 5-7., Sárvár, Magyarország.
2. **Lilla Makszin**, Anikó Kilár, Annamária Bui, Zoltán Szabó, Ágnes Dörnyei, Viktor Farkas, Béla Kocsis, Ferenc Kilár: Fast and extremely sensitive detection of bacterial endotoxins in microchip electrophoresis, 23rd International Symposium on MicroScale Bioseparations, February 1-5, 2009, Boston, USA.
3. **Lilla Makszin**, Anikó Kilár, Krisztina Kovács, Béla Kocsis, Ferenc Kilár: Microchip electrophoresis for fingerprinting endotoxin chemotypes from whole-cell lysates, 6th International Interdisciplinary Meeting on Bioanalysis, November 6-7, 2009, Pécs, Hungary
4. **Lilla Makszin**, Anikó Kilár, Krisztina Kovács, Béla Kocsis, Ferenc Kilár: Microchip electrophoresis for fingerprinting endotoxin chemotypes from whole-cell lysates, 25th International Symposium on MicroScale Bioseparations, March 21-25, 2010, Prague, Czech Republic.
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14. **Lilla Makszin**, Ágnes Blaskó, Pál Gróf, Ferenc Kilár, Béla Kocsis: Endotoxin detection by microchip electrophoresis and EPR in blood, ITP 2013 - 20th International Symposium on Electro- and Liquid Phase- Separation Techniques, October 6-9, 2013, Tenerife, Canary Islands (Spain)
15. **Lilla Makszin**, Ágnes Blaskó, Zoltán Péterfi, Zoltán Berente, Ferenc Kilár, Béla Kocsis: Structure of colitose-containing O-polysaccharides from the lipopolysaccharides of *Proteus morganii* O34 (8662/64), *Escherichia coli* O111 and *Salmonella enterica sv. Adelaide* O35, 30th International Symposium on MicroScale Bioseparations, 27 April - 1 May, 2014, Pécs, Hungary

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