Final report **OTKA 100667**

Lipopolysaccharidomics – a new area
(Lipopoliszacharidomika - egy új terület)

Lipopolysaccharides (LPSs, endotoxins) are essential components of the outer cell membrane of Gram-negative bacteria and play an important role in a number of diseases of bacteria, including Gram-negative sepsis. The hydrophilic carbohydrate part of LPSs consists of a core oligosaccharide (in the case of an R-type LPS) linked to an O-polysaccharide chain (in the case of an S-type LPS), which is responsible for O-specific immunogenicity. The hydrophobic lipid A anchor is composed of a phosphorylated diglucosamine backbone to which varying numbers of ester- and amide-linked fatty acids are attached and this part of the LPSs is associated with endotoxicity. The detailed chemical characterization of endotoxins requires long-lasting large-scale isolation procedures, by which high-purity LPSs can be obtained. However, when large number of bacterial samples and their LPS content are to be compared prompt, small-scale isolation methods are used for the preparation of endotoxins directly from bacterial cell cultures. The purity of the endotoxins extracted by these methods may not be high, but it is sufficient for analysis.

A novel, fast, and sensitive microchip electrophoresis method was developed to analyse and differentiate the smooth (*S*) and rough (*R*) type bacterial endotoxin components labelled covalently with a fluorescent dye. The quantitative analysis of purified lipopolysaccharides, or partially purified samples from whole-cell lysates becomes possible with this method. Two groups with three sub-groups in the first group of *S*-type lipopolysaccharides can be classified based on the electrophoretic profiles. The LOD of the endotoxins from *S*- and *R*-type Gram-negative bacteria was found to be 2.6 ng and 6.9 ng, respectively. This method is capable to replace the commonly used SDS-PAGE combined with silver staining.

The structure of the oligosaccharide repeating units of endotoxins from Gram-negative bacteria is characteristic for the different serogroups and serotypes of bacteria. Detailed examination of the cross-reactions of three enterobacterial serotypes, *Proteus morganii* O34, *Escherichia coli* O111, and *Salmonella enterica sv. Adelaide* O35, was performed using sensitive tests (ELISA, immunoblotting). Fine differences between the endotoxins of the bacteria were detected using silver staining of SDS-PAGE gels and chip-technology for the intact lipopolysaccharides (LPSs). The compositions of the *O*-specific polysaccharides of LPSs extracted from the bacteria were studied, and it was proven that the three cross-reacting bacteria contain *O*-antigens built from the same monosaccharides, namely colitoses linked to glucose, galactose, and *N*-acetyl-galactosamine. The NMR and GC-MS studies revealed that the most probable component for the cross-reaction is the rare sugar, colitose.

We have described a fast and easy micromethod suitable for extracting small quantities of R-type LPSs and a slightly modified micromethod for detection of the lipid A constituents of the LPSs from bacteria grown in different culture media and evaluate the structures with mass spectrometry. The cellular LPSs and lipid A were obtained from crude isolates of heat-killed cells, which were then subjected to matrix-assisted laser desorption/ionization mass spectrometry analysis. The observed ions in the 10-colony samples were similar to those detected for purified samples. The total time for the sample preparation and the MS analysis is less than three hours.

Non-phosphorylated lipid A species confer reduced inflammatory potential for the bacteria. Knowledge on their chemical structure and presence in bacterial pathogens may contribute to the understanding of bacterial resistance and activation of the host innate immune system. In our studies, we reported the fragmentation pathways of negatively charged, non-phosphorylated lipid A species under low-energy collision-induced dissociation conditions of an electrospray ionization quadrupole time-of-flight instrument. Charge-promoted consecutive and competitive eliminations of the acyl chains and cross-ring cleavages of the sugar residues were observed. The A-type fragment ion series and the complementary X-type fragment(s) with corresponding deprotonated carboxamide(s) were diagnostic for the distribution of the primary and secondary acyl residues on the non-reducing and the reducing ends, respectively, of the non-phosphorylated lipid A backbone. Reversed-phase liquid chromatography in combination with negative-ion electrospray ionization quadrupole time-of-flight tandem mass spectrometry could provide sufficient information on the primary and secondary acyl residues of a non-phosphorylated lipid A. As a standard, the hexa-acylated ion at m/z 1636 with the Escherichia coli-type acyl distribution (from E. coli O111) was used. The method was tested and refined with the analysis of other non-phosphorylated hexa- and several hepta-, penta-, and tetra-acylated lipid A species detected in crude lipid A fractions from E. coli O111 and Proteus morganii O34 bacteria.

We have established a new reversed phase-high performance liquid chromatography method combined with electrospray ionization quadrupole time-of-flight tandem mass spectrometry for the simultaneous determination and structural characterization of different lipid A types in bacteria (Escherichia coli O111, Salmonella adelaide O35 and Proteus morganii O34) showing serological cross-reactivity. The complex lipid A mixtures (obtained by simple extraction and acid hydrolysis of the outer membrane lipopolysaccharides) were separated and detected without phosphate derivatization. Several previously unidentified ions were detected, which differed in the number and type of acyl chains and number of phosphate groups. In several cases, we observed the different retention of isobaric lipid A species, which had different secondary fatty acyl distribution at the C2′ or the C3′ sites. The fragmentation of the various, C4′ monophosphorylated lipid A species in deprotonated forms provided structural assignment for each component. Fragmentation pathways of the tri-acylated, tetra-acylated, pentaacylated, hexa-acylated and hepta-acylated lipid A components and of the lipid A partial structures are suggested. As standards, the hexa-acylated ion at m/z 1716 with the E. coli-type acyl distribution and the hepta-acylated ion at m/z 1954 with the Salmonella-type acyl distribution were used. The results confirmed the presence of multiple forms of lipid A in all strains

analyzed. In addition, the negative-ion mode MS permitted efficient detection for non-phosphorylated lipid A components, too.

An official invitation has arrived to our group to write a detailed review about endotoxins in the prestigious journal: Mass Spectrometry Reviews. The focus of the review was the application of mass spectrometry to the structural characterization of bacterial lipopolysaccharides (LPSs), also referred to as ‘‘endotoxins,’’ because they elicit the strong immune response in infected organisms. Recently, a wide variety of MS-based applications have been implemented to the structure elucidation of LPSs. Methodological improvements, as well as on- and off-line separation procedures, proved the versatility of mass spectrometry to study complex LPS mixtures. Special attention was given in the review to the tandem mass spectrometric methods and protocols for the analyses of lipid A, the endotoxic principle of LPS. We compared and evaluated the different ionization techniques (MALDI, ESI) in view of their use in intact R- and S-type LPS and lipid A studies. Methods for sample preparation of LPS prior to mass spectrometric analysis were also described. The direct identification of intrinsic heterogeneities of most intact LPS and lipid A preparations is a particular challenge, for which separation techniques (e.g., TLC, slab-PAGE, CE, GC, HPLC) combined with mass spectrometry are often necessary. A brief summary of these combined methodologies to profile LPS molecular species has also been provided.

For a proper investigation of charged lipopolysaccharides, having amphoteric properties, we started to investigate and develop a suitable isoelectric focusing method, which is able to detect components with mass spectrometry.

CIEF of components following sequential injection of ampholytes and the sample zone offers unique advantages for analysis. The most important one of these is the efficient separation of amphoteric compounds having p*I*s outside the pH range of the ampholytes applied, but the resolution of the components can be increased by an adequate setup in the injection protocol. In our studies, the effect of the pH of the anolyte and catholyte on the selectivity and speed of the isoelectric focusing was investigated. Changes in the pH values significantly influenced the resolution and the length of the pH gradient, while changes in the charge state of components were also observed. Three ampholyte solutions (from different suppliers) covering only two pH units were used for the analyses of substituted nitrophenol dyes in uncoated capillary. With appropriate setup, the components, with p*I*s not covered by the ampholyte pH range, migrated in charged state outside the pH gradient. This phenomenon is preferable for coupling isoelectric focusing to MS detection, by evading the undesirable ion suppression effect of ampholytes.

The impact of initial sample distribution on separation and focusing of analytes in a pH 3– 11 gradient formed by 101 biprotic carrier ampholytes under concomitant electroosmotic displacement was studied by dynamic high-resolution computer simulation. Data obtained with application of the analytes mixed with the carrier ampholytes (as is customarily done), as a short zone within the initial carrier ampholyte zone, sandwiched between zones of carrier ampholytes, or introduced before or after the initial carrier ampholyte zone were compared. With sampling as a short zone within or adjacent to the carrier ampholytes, separation and

focusing of analytes is shown to proceed as a cationic, anionic, or mixed process and separation of the analytes is predicted to be much faster than the separation of the carrier components. Thus, after the initial separation, analytes continue to separate and eventually reach their focusing locations. This is different to the double-peak approach to equilibrium that takes place when analytes and carrier ampholytes are applied as a homogenous mixture. Simulation data reveal that sample application between two zones of carrier ampholytes results in the formation of a pH gradient disturbance as the concentration of the carrier ampholytes within the fluid element initially occupied by the sample will be lower compared to the other parts of the gradient. As a consequence thereof, the properties of this region are sample matrix dependent, the pH gradient is flatter, and the region is likely to represent a conductance gap (hot spot). Simulation data suggest that sample placed at the anodic side or at the anodic end of the initial carrier ampholyte zone are the favorable configurations for capillary isoelectric focusing with electroosmotic zone mobilization.

The behaviour of sample components whose p*I* values are outside the pH gradient established by 101 hypothetical biprotic carrier ampholytes covering a pH 6–8 range was investigated by computer simulation under constant current conditions with concomitant constant electroosmosis toward the cathode. Data obtained with the sample being applied between zones of carrier ampholytes and on the anodic side of the carrier ampholytes were studied and found to evolve into zone structures comprising three regions between anolyte and catholyte. The focusing region with the pH gradient is bracketed by two isotachopheretic zone structures comprising selected sample and carrier components as isotachophoretic zones. The isotachophoretic structures electrophoretically migrate in opposite direction and their lengths increase with time due to the gradual isotachophoretic decay at the pH gradient edges. Due to electroosmosis, however, the overall pattern is being transported toward the cathode. Sample components whose p*I* values are outside the established pH gradient are demonstrated to form isotachophoretic zones behind the leading cation of the catholyte (components with p*I* values larger than 8) and the leading anion of the anolyte (components with p*I* values smaller than 6). Amphoteric compounds with appropriate p*I* values or nonamphoteric components can act as isotachophoretic spacer compounds between sample compounds or between the leader and the sample with the highest mobility. The simulation data obtained provide for the first time insight into the dynamics of amphoteric sample components that do not focus within the established pH gradient.

Further computer simulation studies dealt with carrier ampholyte based CIEF in presence of narrow pH gradients. With this technique, amphoteric sample components with pI values outside the pH gradient are migrating isotachophoretically toward the cathode or anode whereas components with pI values within the gradient become focused. In order to understand the processes occurring in presence of the electric field, the behavior of both carrier ampholytes and amphoteric sample components is investigated by computer modeling. Characteristics of two pH unit gradients with end components having pI values at or around 7.00 and conditions that lead to the formation of a water zone at neutrality were investigated. Data obtained reveal that a zone of water is formed in focusing with carrier ampholytes when the applied pH range does not cover the neutral region, ends at pH 7.00 or begins at pH 7.00. The presence of

additional amphoteric components that cover the neutrality region prevent water zone formation under current flow. This situation is met in experiments with narrow pH gradients that end or begin around neutrality. Simulation data reveal that no water zone evolves when atmospheric carbon dioxide dissolved in the catholyte causes the migration of carbonic acid (in the form of carbonate and/or hydrogen carbonate ions) from the catholyte through the focusing structure. An electrolyte change in the electrode solution does not have an impact on the focusing part but does change the isotachophoretic pattern migrating behind the leading ion.