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Bacterial Carbohydrate Antigens: From Primary Structure to Non-covalent Interactions

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Two structural aspects of bacterial antigens studied by mass spectrometry are presented in this contribution: identification of unusual modifications in the carbohydrate chains in the case of lipoteichoic acid from *Streptococcus pneumoniae* and determination of non-covalent carbohydrate/protein complexes of *Salmonella* oligosaccharide antigen with natural and/or recombinant antibody proteins. The ability of mass spectrometric strategies for detailed analysis of the diversity of carbohydrate chain structures in the living nature is demonstrated here by three modern desorption techniques, fast atom bombardment, matrix-assisted laser desorption and electrospray, offering the possibility for sequencing and for detection of molecular mass of intact large molecules and their non-covalent complexes at high sensitivity. The general understanding of carbohydrate recognition in biological systems at the molecular level in sense of the Emil Fischer's Schloss/Schlüssel principle is the ultimate goal of this strategy.

PROLOGUE

In Zürich, the autumn mornings can be grey, foggy and humid, as it was in autumn 1969. The streetcar number 9 would take you on its route direction Bahnhof Enge, then Bellevue square, Rämistraße uphill. If you were working at the Chemisches Institut der Universität Zürich, like me at that time, you would have to get out at the station after Schauspielhaus. The way to the laboratories of Eidgenössische Technische Hochschule is longer,

* Dedicated to Professor Vlado Prelog on the occasion of his 90th birthday.

two stations further on. Professor Prelog was taking the same streetcar line number 9 and I would meet him during the morning drives now and then. We had some conversation in Croatian, a rare opportunity for me after leaving my native Zagreb. One morning in the streetcar, after hesitating a while, I seized the opportunity to pose some direct questions on topic which puzzled us a lot. Why would a famous organic chemist get involved with microorganisms for organic transformations? Prelog's answer was a short and simple statement on stereospecificity of chemical reactions performed by use of bacteria *versus* those of chemical reagents.[1] For a young ongoing chemist on this grey early morning, it was hard to accept the idea of sophisticated concepts for chemical reactions originated in human mind in which bacteria may be playing a serious role.

Now, more than twenty five years later it is a trace of memory, which inspired me to catch up with the keyword »bacteria«. Working in field of glycobiology, my contribution for Professor Prelog's nintieth anniversary is giving another view from another angle. This part of our work on structure and function of complex carbohydrates is represented by a report on structure and interactions of bacterial antigens.
Let's talk about bacteria!

INTRODUCTION

Biochemistry and molecular biology have so far been principally concerned with the pursuit of the unity throughout bacteria to animal success, culminating in the proposal of the so-called central dogma, DNA/RNA/protein, represented by two unique types of chain molecules, nucleic acids and proteins. A third unique chain molecule in living nature is the carbohydrate chain, characterized by diversity of its structures, as illustrated by a calculation: a trimer of sugar unit, a trisaccharide composed of different sugars XYZ, theoretically gives rise to 1056 structural isomers, compared to 6 of a peptide. Possible involvement of carbohydrate chain structures in the diversity of living nature, varying from species, individuals, organs, tissues, and up to cells, can be envisaged by perception of differences from normal, to pathological (cancer, metastasis) and in the stages of development (embryonic differentiation) structural patterns. The surface of the cells is largely covered with various carbohydrate chains, thus being involved in the cell to cell interactions. Since Heidelberger and Avery (1923) the awareness on association of bacterial antigens with carbohydrate structure was established. Bacterial cells and certain viruses utilize carbohydrate-mediated cell recognition and adhesion as an initial step for infection. Recent observations suggest that either in a particular cell group *in vivo* like a tissue or in an isolated cultured situation is different from each other and that essential function of the carbohydrate chain is realized in such grouping of cells or

the society of cells. Thus, glycobiology is aiming at finding the principle of cell sociology or of the self organizing multi-cellular system related to the forming process of two millions of classified or manyfold of that non-classified species currently on earth.[2]

Since its beginnings mass spectrometry (»spectrometry of mass« according to F. W. Aston) demonstrated its ability to contribute essential knowledge and understanding in many areas of science. In its early days the measurements of atomic masses and isotopic abundances, performed on magnetic sector instruments with increasing accuracy, were completed even before the discovery of neutrons.[3] In recent times, mass spectrometry became an efficient tool to study macromolecular aspects of cell biology and medicine, for the normal and impaired cellular functions and cell interactions. The detection of molecular ions and the sequencing of macromolecules were two general goals approached during the last decade. The structural changes in the life of a protein, a nucleic acid or a glycoconjugate since its biosynthesis to its degradation can now be studied and described on the molecular level. Current ability to detect even extreme high masses up to 1 million Da by MALDI-TOF-MS as recently reported from two IgM species,[4] could become an inspiration for new types of supramolecular analyses, as for studies of structural assemblages of cell organelles. Two aspects of mass spectrometry in glycobiology of bacterial antigens will be presented in this contribution. The ability of mass spectrometric strategies for detailed analysis of carbohydrates modified by rare functional groups and for sequencing of oligomers using subpicomol amounts of material will be stressed out in the case of lipoteichoic acid from *Streptococcus pneumoniae*. Detection of non-covalent complexes in the gas phase by electrospray mass spectrometry opened new options for direct studies of non-covalent interactions in general, as presented here for the *Salmonella* carbohydrate antigen with natural and/or recombinant antibody proteins.

LIPOTEICHOIC ACID OF *Streptococcus pneumoniae*

The Gram-positive bacterium *Streptococcus pneumoniae* is a major cause of pneumonia, sepsis and meningitis. Although the invasive disease is severe, some 40% of individuals harbour the pneumococcus in the nasopharynx asymptomatically.[5] The virulence, causing the attachment to the G-protein coupled platelet-activating factor (PAF) receptor was recently found to be related to the phosphorylcholine, present in the pneumococcal cell wall, as a determinant of proinflammatory activity.

A choline-containing lipoteichoic acid, associated with the plasma membrane *via* covalently attached fatty acids, was the first identified »lipocarbohydrate« among Gram-positive bacteria,[6] whereas its structural relation to the pneumococcal C polysaccharide, teichoic acid, remained largely un-

clear. By serological methods, all 83 known types of *Streptococcus pneumoniae* were shown to possess same antigens, those of the polysaccharide C of teichoic acid and the Forssman antigen of lipoteichoic acid. Both were shown to induce the meningeal inflammation[7] but only the lipoteichoic acid to activate the alternate complement pathway.[8]

As a result of our recent investigations on surface determinants in the pneumococcal cell wall by using a combination of specific chemical degradation and derivatization reactions and fast atom bombardment (FAB)- and matrix-assisted laser desorption ionization (MALDI)-mass spectrometry, the detailed structure of lipoteichoic acid was revealed.[9] Its skeleton was found to be represented by an oligomeric carbohydrate chain, containing pentasaccharide repeating units, linked to each other by phosphodiester bonds and to the glycolipid membrane anchor of trihexosyldiacylglycerol. 2-acetamido-4-amino-2,4,6-trideoxygalactose (AATGal), a characteristic component of the teichoic acid, was found as a constituent in the repeating unit, which was additionally modified by two phosphocholines. In the glycolipid moiety, obtained from lipoteichoic acid after hydrolysis by 48% aquous HF, and investigated in form of its *N*-deuteroacetylated derivative by (+)FAB-MS, a trisaccharide Glc*p*ß(1-3)AATGal*p*ß(1-3)Glc*p* was found to be linked to a diacylglycerol anchor. Phosphocholine moieties modify the carbohydrate chain via substitutions at C-6 of both adjacent *N*-acetylgalactosamine (GalNAc) units (Figure 1).

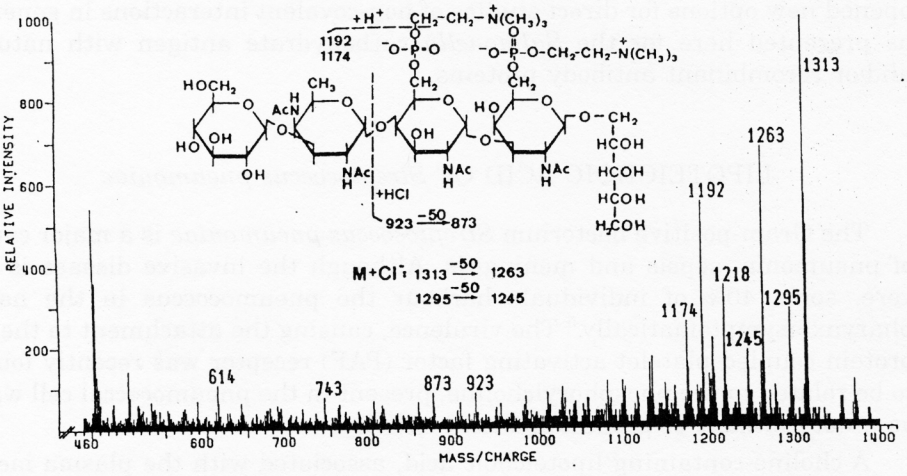
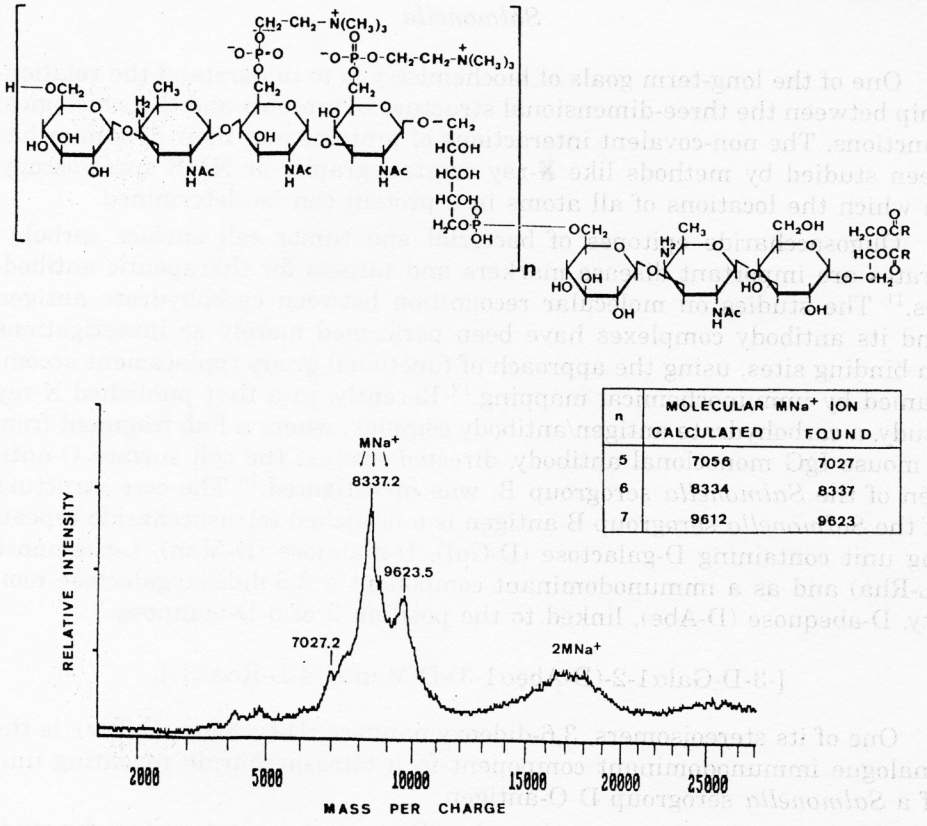


Figure 1. Negative ion FAB-MS and structure of the *N*-acetylated bis(phosphocholine)-carrying repeating unit of lipoteichoic acid from *Streptococcus pneumoniae.*



n	MOLECULAR CALCULATED	MNa+ ION FOUND
5	7056	7027
6	8334	8337
7	9612	9623

Figure 2. Positive ion MALDI-MS of the de-*O*-acylated and *N*-acetylated pneumococcal lipoteichoic acid and its overall structure. Oligosaccharide concentration (a) 5 pMol/ml (b) 10 pMol/ml.

Oligomer distribution in different lipoteichoic acid samples was investigated by positive ion MALDI-MS. In the main fraction a lipid anchor-linked hexamer (33 monosaccharides *in toto*) was identified as a major species (Figure 2).

An interesting link to the already existing data from immunochemical experiments was found in a subsequent structural reinvestigation of carbohydrate chains from streptococcal teichoic acid (C-polysaccharide).[10] Using the same approach, its structure was shown to be identical with this of the lipoteichoic acid, but, instead to the glycolipid anchor, the carbohydrate chain is covalently linked to the peptidoglycan layer of the cell wall. A possible common biosynthetic precursor for both glycoconjugates, an unique feature among Gram-positive bacteria, should be now discussed.

CARBOHYDRATE ANTIGEN/ANTIBODY INTERACTIONS IN *Salmonella*

One of the long-term goals of biochemistry is to understand the relationship between the three-dimensional structure of proteins and their biological functions. The non-covalent interactions of proteins and their dynamics has been studied by methods like X-ray crystallography or NMR spectroscopy, in which the locations of all atoms in a protein can be determined.

Oligosaccharide epitopes of bacterial and tumor cell surface carbohydrates are important disease markers and targets for therapeutic antibodies.[11] The studies on molecular recognition between carbohydrate antigen and its antibody complexes have been performed merely as investigations on binding sites, using the approach of functional group replacement accompanied by immunochemical mapping.[12] Recently, in a first published X-ray study, a carbohydrate antigen/antibody complex, where a Fab fragment from a mouse IgG monoclonal antibody, directed against the cell surface O-antigen of the *Salmonella* serogroup B, was investigated.[13] The core structure of the *Salmonella* serogroup B antigen is a branched tetrasaccharide repeating unit containing D-galactose (D-Gal), D-mannose (D-Man), L-rhamnose (L-Rha) and as a immunodominant component a 3,6-dideoxygalactose moiety, D-abequose (D-Abe), linked to the position 3 of a D-mannose:

$$[\text{-3-D-Gal}\alpha\text{1-2-(D-Abe}\alpha\text{1-3)-D-Man}\alpha\text{1-4-L-Rha}\alpha\text{1-}]_n$$

One of its stereoisomers, 3,6-dideoxymannose (D-tyvelose, D-Tyv), is the analogue immunodominant component in a tetrasaccharide repeating unit of a *Salmonella* serogroup D O-antigen.

Electrospray mass spectrometry has become an important tool for studies of non-covalent binding, due to its ability to determine the stoichiometry of non-covalent complexes in the gas-phase directly from the mass shifts of molecular ions.[14]

In the ESI-MS experiment a group of multicharged molecular ions arising after protonation of a *Salmonella* antibody Fab fragment was observed, showing some heterogeneity in the protein size, possibly due to the conditions of papain digestion of the antibody protein, used to obtain the Fab fragment. The ions at the charge states (+12) at m/z = 2190.4 to (+9) at m/z = 2921.0 (Figure 3), corresponding to the M_r of 46 853.1 Da after deconvolution, were chosen to observe the process of complexation with carbohydrate antigens. After addition of a carbohydrate ligand, the hexadecasaccharide $[\text{-3-D-Gal}\alpha\text{1-2-(D-Abe}\alpha\text{1-3)-D-Man}\alpha\text{1-4-L-Rha}\alpha\text{1-}]_4$, the protein molecular ions decreased giving rise to the ions originating from the oligosaccharide/protein complex formation in 1:1 stoichiometry, at the M_r of 49 272.2 Da (after deconvolution) (Figure 4). The binding of the recombinant single chain antibody protein was documented by analogue experiment. No shifts of molecular ions were observed when an O-antigenic determinant

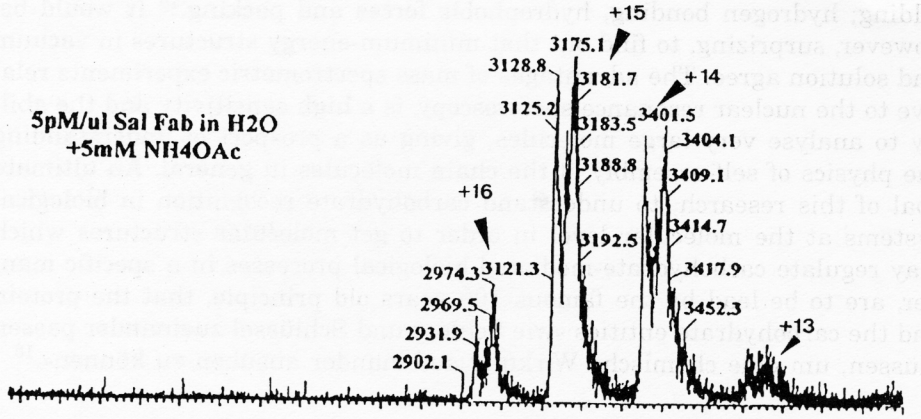


Figure 3. Positive ion ESI-MS of the *Salmonella* antibody Fab fragment and the charge attribution of the molecular ions in cluster. After deconvolution its molecular mass was determined to be 46 851.9 Da.

of the *Salmonella* serogroup D, the dodecasaccharide [-3-D-Galα1-2-(D-Tyvα1-3)-D-Manα1-4-L-Rhaα1-]$_3$, was used instead.[14]

Using new generation of instruments the accesss for probing non-covalent structural features of proteins by mass spectrometry became available, leading to an explosive rate of experiments in order to establish comprehensive and clear rules for behaviour of macromolecules in the gas-phase. Historically three major forces have been thought to be important in protein

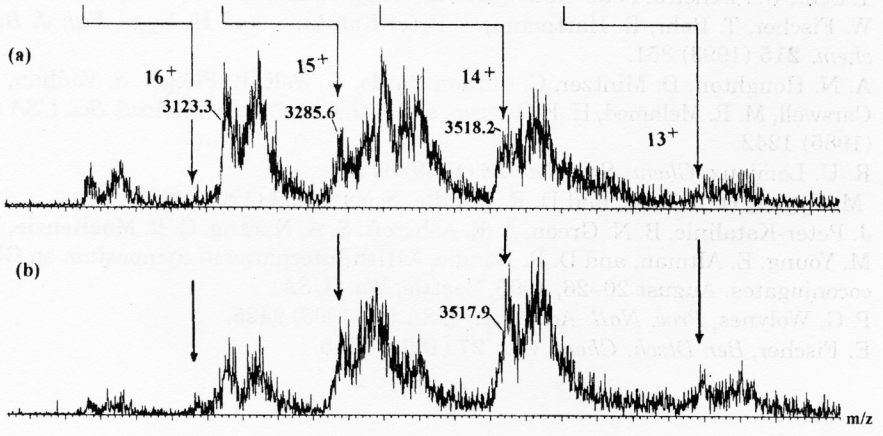


Figure 4. Positive ion ESI-MS of the *Salmonella* hexadecasaccharide/antibody Fab fragment. (a) Oligosaccharide concentration 5 pMol/μl, (b) 10 pMol/μl.

folding; hydrogen bonding, hydrophobic forces and packing.[15] It would be, however, surprizing, to find out that minimum-energy structures in vacuum and solution agree. The advantages of mass spectrometric experiments relative to the nuclear resonance spectroscopy, is a high sensitivity and the ability to analyse very large molecules, giving us a prospect of understanding the physics of self-assembly of the chain molecules in general. An ultimate goal of this research, to understand carbohydrate recognition in biological systems at the molecular level in order to get molecular structures which may regulate carbohydrate-mediated biological processes in a specific manner, are to be lead by the famous 100 years old principle, that the protein and the carbohydrate entities »wie Schloss und Schlüssel zueinander passen müssen, um eine chemische Wirkung aufeinander ausüben zu können«.[16]

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SAŽETAK

Bakterijski antigeni ugljikohidrata: od primarne strukture do nekovalentnih interakcija

Jasna Peter-Katalinić

Prikazana su dva strukturna aspekta bakterijskih antigena proučavanih masenom spektrometrijom: identifikacija neuobičajenih promjena na ugljikohidratnom lancu u primjeru lipoteihoične kiseline iz *Streptococcus pneumoniae* i određivanje nekovalentnog kompleksa antigena ugljikohidrat-protein za oligosaharide *Salmonellae* s prirodnim i/ili rekombinantnim proteinima antitijela. Sposobnost masene spektrometrije da detaljno analizira različite strukture ugljikovodičnih lanaca u živoj prirodi prikazana je s tri moderne desorpcijske tehnike: bombardiranje brzim atomima, laserska desorpcija uz pomoć matrice, i elektrosprej. One pružaju mogućnost za sekvenciranje i određivanje, s velikom osjetljivošću, molekulske mase velikih intaktnih molekula i njihovih nekovalentnih kompleksa. Konačni je cilj te strategije općenito razumijevanje prepoznavanja ugljikohidrata u biološkim sistemima na molekulskoj razini u smislu postavke Emila Fischera brava/ključ.