Posters

P1

HIV-1 PROTEASE MUTATIONS AND INHIBITOR MODIFICATIONS. RESULTS FROM A SERIES OF X-RAY STRUCTURES

T. Skálová, J. Dohnálek, J. Dušková, H. Petroková, J. Hašek

Institute of Macromolecular Chemistry, Czech Academy of Sciences, Heyrovskeho nam. 2, 162 06 Praha 6, Czech Republic. skalova@imc.cas.cz

Two new X-ray structures of (A71V, V82T, I84V) HIV-1 protease – inhibitor complexes were determined. This contribution presents their comparison with previously determined structures. Altogether, there are four pairs of similar peptidomimetic inhibitors [1] – with ethyleneamine [2-3], hydroxyethylene [4] and two with S-hydroxyethylamine isostere [5-6] – each in complex with wild-type and (A71V, V82T, I84V) HIV-1 protease (range of inhibition constants 0.1 – 1000 nM). This series makes it possible to see structural changes caused by protease mutations and inhibitor modifications.

It was found that all inhibitors form a saturated network of hydrogen bonds to both wild-type and mutant protease. Extreme cases of inhibition constants in the series can be explained by structural changes in inhibitor binding caused by inhibitor modifications (mainly the length of the isostere and fit of P_1 and P_1' side chains in the protease binding pockets).

Moreover, some systematic structural changes may be explained as consequences of protease mutations (including effects of mutation of residue 71 which lies ca. 16 Å from active site): 1. Mutant protease-inhibitor complexes show higher conformational strain in inhibitors than in wild-type complexes in all four cases. 2. Three of the four pairs of complexes show a shorter hydrogen bond between the catalytic residues Asp 25 and Asp 125 in the mutant complexes than in the wild-type complexes.

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P2

CRYSTALLIZATION AND PRELIMINARY X-RAY STRUCTURAL ANALYSIS USED FOR CHARACTERISATION OF DI-HEME CYTOCHROME c_4

I. Tomčová and I. Kutá Smatanová

Institute of Physical Biology, University of South Bohemia, Nové Hrady, Czech Republic
Institute of Systems Biology and Ecology, Academy of Sciences of the Czech Republic, Nové Hrady

The cytochromes and the hydrogenases are ubiquitous proteins present in all living organisms and involved in a variety of intracellular processes that are essential for life. Most notable is their participation in electron transfer reactions, usually as components of a complex reaction pathway, necessary for the production of energy either through oxidation of metabolites or via photosynthesis [1]. The cytochromes consist of two heme molecules in single polypeptide chain with classical Cys–X–Y–Cys–His heme binding sites. It is the first cytochrome of its class that comes from an anaerobic organism. Due to their important function, it is of essential interest to study structural features of metalloenzymes using X-ray crystallography.

Cytochrome c_4 (cyt c_4) and hydrogenase from the purple sulphur photosynthetic bacterium *Thiocapsa roseopersicina* were isolated and purified according to [2]. Cyt c_4 was crystallized using standard crystallization methods based on vapor diffusion [3] and advanced crystallization method based on the counter-diffusion [4]. Crystallization trials were performed at 20 °C. The most suitable concentration of protein 10 mg/ml was found. The first suitable crystal growth was observed at pH 6.0 [Figure 1] using the

Fig. 1

addition of metal ions – Cu^{2+}, Cd^{2+}, Co^{2+}, Ba^{2+} (Hampton Research Additive Screen).

Colored crossbred plates of holoprotein crystals with dimensions of approximately 200 x 50 x 30 ěm grew within 3–4 days under several conditions.

The monocrystals of cyt c_4 were tested at the home source diffractometer at LEC (University of Granada) and measured at synchrotron DESY (Hamburg), beamline X11 upto resolution 1.72 Å. Structure of cyt c_4 will be solved using molecular replacement method.

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P3

STRUCTURAL CHARACTERIZATION OF FLAVOPROTEIN WrbA FROM *Escherichia coli* BY USING X-RAY DIFFRACTION ANALYSIS

J. Wolfová[1,2], J. Carey[3] and I. Kutá Smatanová[1,2]

[1]Institute of Physical Biology, University of South Bohemia České Budějovice, Zámek 136, CZ-373 33 Nové Hrady, Czech Republic
[2]Institute of Systems Biology and Ecology, Academy of Science of the Czech Republic, Zámek 136,CZ-373 33 Nové Hrady, Czech Republic
[3]Chemistry Department, Princeton University, Washington Rd and William St, Princeton, NJ 08544-1009, USA

X-ray diffraction analysis is a main tool for structural analysis of biological macromolecules. Using of synchrotron radiation as a source is preferred for X-ray diffraction on protein crystals This study is concerned with the tryptophan (W)-repressor binding protein A (WrbA) from *Escherichia coli*, which belongs to a new family of multimeric flavodoxin-like proteins implicated in oxidative-stress defense. The suggested function is in agreement with the finding that the WrbA protein can be expressed also under the stress conditions [1]. The WrbA protein obtained its name with reference to its reported effect on the binding interaction of tryptophan repressor [2]. Nevertheless, it was shown later, that WrbA doesn't influence the binding specifically [3], and thus its function in the living cells remains unclear. Based on the computational studies and biochemical experiments it was shown that the WrbA protein shares the open, twisted / fold with flavodoxins and its physiological cofactor is the flavin mononucleotide (FMN) as well [3, 4]. Unlike canonical flavodoxins the WrbA protein displays some interesting structural properties representing the new family, namely the structure of the flavin-binding pocket and multimerization. Structural characterization of the new protein family together with the understanding of the physiological role of WrbA protein in living organisms motivated our research of the WrbA protein using X-ray diffraction techniques. Moreover structural similarity with the proteins of the known functions (electron transfer in metabolic processes [8], protective effects on cells against free radicals [5-7], activation of cytotoxic drugs in cancer chemotherapy [9]) indicates the potential usage of the WrbA protein in pharmaceutics or medicine.

The pure WrbA apoprotein (protein without FMN) as well as the WrbA protein in complex with FMN were crystallized using standard vapor diffusion methods and counter-diffusion methods in single capillaries [10]. Optimization of crystallization conditions by using additives, especially Cd^{2+} and Li-citrate, led to getting single WrbA apoprotein crystals suitable for diffraction measurements [11]. The crystals diffracted to a resolution of 2.2 Å at synchrotrons DESY (X13) in Hamburg (Germany), and Elletra (XRD1) in Trieste (Italy), but the diffraction data were not sufficiently good for solving structure. Additional optimization of crystal growth or preparation of the WrbA apoprotein crystals for diffraction analysis is necessary to obtain the data for solving the 3D-structure. The WrbA protein in complex with FMN showed better crystallizability then the protein without cofactor. This demonstrates the influence of cofactors on crystallization properties of proteins. Two crystallization conditions were found for this protein, both different from those used in the case of the WrbA apoprotein. The crystals of the liganded protein diffracted up to a resolution of 2.0 Å at synchrotron DESY (X13) in Hamburg (Germany). The data sets collected at synchrotron were processed and the solving of the structure is in progress.

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P4

CRYSTAL STRUCTURE OF PHOTOSYSTEM II FROM *Thermosynechococcus elongatus* REFINED BY COMPUTATIONAL METHODS

Peter Palenčár[1], František Vácha[1,2], and Michal Kutý[1,2]

[1]*Institute of Physical Biology, University of South Bohemia, Zámek 136, 37333 Nové Hrady, Czech Republic*
[2]*Institute of Systems Biology and Ecology, AS CR,Zámek 136, 37333 Nové Hrady, Czech Republic*
[3]*Institute of Plant Molecular Biology, AS CR, Branišovská 31, 370 05 České Budějovice, Czech Republic*

Crystal and NMR structures are essential and fundamental in performing almost all molecular modelling techniques. Three dimensions resolution of such structures is certainly one of the most crucial criteria of quality and credibility. Researchers made great effort to prepare crystals of photosystem II (PS II) from algae and higher plants in the last decades. However, till now there are only two experimental crystal structures resolved at adequate resolution. Both are from the same common organism *Thermosynechococcus elongatus*. First was obtained at 3.5 Å (PDB code: 1S5L) [1] and second at 3.2 Å (PDB code: 1W5C) [2] overall resolution. By performing series of molecular dynamics (MD) simulations at appropriate time scales also coupled partially with quantum-chemical calculations, it is possible to increase the model accuracy mainly in the regions, where the probability of spatial orientation of amino acid side chain lacks appropriate electron density or other sources of experimental data. We present here more natural-like, geometrically - optimised structures of extended reaction centre (RC) of PS II.

Recently, changes in excitonic interactions of PS II RC pigments upon light-induced oxidation of primary donor (P680) or reduction of primary acceptor pheophytin *a* (Phe *a*), were analysed using absorption and circular dichroism (CD) spectra [3, 4]. In contrast to the oxidation of primary donor, the light-induced change in the CD spectrum upon primary acceptor reduction was temperature-dependent. This suggests a hypothesis that at a room temperature the reduced Phe *a* induces conformational changes of the RC protein environment, which affects the excitonic interaction of the RC chlorophylls. Having optimised structural models of PS II RC we were able to elucidate and describe some of the details of these processes.

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P5

STRUCTURE – FUNCTION STUDIES OF RS20L LECTIN FROM THE BACTERIUM *Ralstonia solanacearum*

Nikola Kostlánová[1], Edward Mitchell[2], Ondřej Šulák[1], Nechama Gilboa-Garber[3], Anne Imberty[4] a Michaela Wimmerová[1,5]

[1]National Centre for Biomol. Research & [5]Dept. of Biochemistry, Masaryk University, Brno, Czech Republic
[2]E.S.R.F Experiment Division, BP220, F-38043 Grenoble cedex,France
[3]CERMAV-CNRS, BP 53, F-38041 Grenoble cedex 09, France
[4]Bar-Ilan University, Faculty of Life Science, Ramat Gan 52900, Israel

Lectins are sugar-binding proteins of non-immunoglobulin nature that agglutinates cells or precipitates glycoconjugates. Their specificity is usually defined by the monosaccharides or oligosaccharides that are best at inhibiting the agglutination or precipitation the lectin causes. Lectins are of interest because of their wide variety of properties and potential applications (pharmacology, immunology, cancer therapy, agriculture...).

The comprehension of the molecular mechanisms, which gives a pathogenic bacterium the ability to invade, colonize and reorient the physiopathology of its host is a goal of primary importance and such studies may direct the conception of new strategies to fight against these pathogenic agents.

Ralstonia solanacearum is a bacterial pathogen, which causes a wilt disease in several economically important agricultural crops, such as potatoes, tomatoes, peppers, eggplant, banana... [1] Causes of this disease are known as Southern wilt, bacterial wilt, and brown rot of potato. The disease is transmitted through soil, contaminated water, equipment, personnel, and by transplanting infected plants. It is not spread through the air, from plant to plant through the splashing of water. The bacterium *R. solancearum* is a widely accepted model organism for the study of pathogenicity in plants.

Until our knowledge now, the *R. solanacearum* bacterium has been producing three soluble lectins. RSL (MW 9900), which exhibits sugar specifity to L-fucose [2] and partial sequence homology to mushroom Aleuria aurantia lectin AAL [3], RS-IIL (MW 11601) lectin [4] resembles PA-IIL from human pathogen *Pseudomonas aeruginosa* in structure and properties but differs in sugar specifity [5]. The last one is RS20L (MW 20034), which displays mannose and xylose binding ability.

This short communication structurally and functionally describes RS20L, a 20kDa lectin from *R. solanacearum*, which has no sequence similarity to any known lectin amino acid sequence, but resolution of crystal structure showed high structural similarity to animal galectins.

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P6

STRUCTURAL STUDIES OF M75 FAB FRAGMENT IN COMPLEX WITH ITS EPITOPE PEPTIDE

P. Mader, R. Š ouračová, J. Brynda, J. Závada

Institut of Molecular Genetics, Academy of Science of the Czech Republic, Flemingovo nám. 2, CZ - 166 37 Prague 6, Czech Republic

The poster describes structural elements and thermodynamics of association of an antibody, M75, to an epitope peptide derived from its protein antigen, proteoglycan-like (PG) segment of human carbonic anhydrase IX (CA IX). Among fourteen human carbonic anhydrases, only the CA IX isoform is strongly associated with certain types of cancer. A unique structural feature of CA IX is its extra component - the proteoglycan-like (PG) segment, located at the amino terminus of the molecule. Conceivably, this segment could be of relevance for oncogenesis, therefore it deserves a more detailed investigation.

The PG amino acid sequence [Q16790; gi:5915865] comprises 59 residues:

SSGEDDPL**GEEDLP**SEEDSPREEDPP**GEEDLPGEED LPGEEDLP**EVKPKSEEEGSLKL

A remarkable feature of the PG segment of the CA IX molecule is a high content of dicarboxylic amino acids (27 D + E out of total 59 residues) and a low content of basic ones (4 R + K). Most of the dicarboxylic amino acids are grouped in four identical repeats of the motif GEEDLP (bold) and in its three modified versions. The epitope peptide used in this study (underlined) seems to be an adequate structural representation of the protein antigen since predictions show lack of any secondary structure in the PG domain. Abnormal expression of CA IX in various commonly occurring carcinomas suggests its involvement in oncogenic pathways. CA IX is also a cell adhesion molecule (CAM) that can mediate attachment of cells to non-adhesive solid support [1]. For a secondary structure-lacking, flexible peptide it might be intuitively expected that such ligand would adopt a complementary shape and undergo stabilization in the complex with its cognate antibody: this would be accompanied with a loss of the conformational freedom and with an unfavorable entropy contribution, measurable with microcalorimetry methods. We show here that upon the epitope peptide binding a substantial structural re-arrangement occurs also in one of the antibody hypervariable loops and that the accompanying local stabilization can be traced in comparisons of the crystal structures of the free and complexed antibody. Somewhat unexpectedly, all substantial 3D structural transitions occur in the hypervariable loops other than those that form counterparts of the epitope dicarboxylic amino acid residues and that provide for major enthalpy contributions.

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P7

STRUCTURAL CHARACTERIZATION OF 2-BROMO-*ACI*-ERGOKRYPTININE

B. Klepetářová,[1] J. Čejka,[1] B. Kratochvíl,[1] L. Cvak,[2] A. Jegorov[3,*]

[1]Department of Organic Structure Analysis, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo n. 2, 166 10 Praha 6, Czech Republic
[2]IVAX Pharmaceuticals, Ostravska 29, 747 70 Opava, Czech Republic
[3]IVAX Pharmaceuticals, Research Unit, Branisovska 31, 370 05 Ceské Budejovice, Czech Republic

Ergot alkaloid bromocriptine (2-bromo- -ergokryptine) exhibits several therapeutic effects - it is used in the treatment of Parkinson's disease, migraine and it is a strong inhibitor of prolactin formation [1]. It is used in the form of its mesylate salt. With regard to its importance, considerable effort has been devoted to its degradation products as potential impurities of the drug substance.

2-bromo-*aci*-ergokryptinine is one of the possible degradation products of bromocriptine mesylate active substance. It is formed by a combination of two degradation reactions - isomerization in position 8 of ergoline part and isomerization in position 2´ of the peptidic moiety. Whereas isomerization in position 8 of ergoline part is fairly well documented, any crystal structure determination of *aci*-ergopeptine has apparently not been reported yet.

Two solvatomorphs of 2-bromo-*aci*-ergokryptinine (bis-acetone solvate and bis-2-butanone solvate) were prepared and their structures were determined by single crystal X-ray diffraction. Their molecular conformations were compared with the structure of active substance bromocriptine mesylate [2].

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P8

SIMULATION OF THE LIPID MEMBRANE WITH PYRENE PROBE IN THE GEL AND LIQUID CRYSTALLINE PHASE

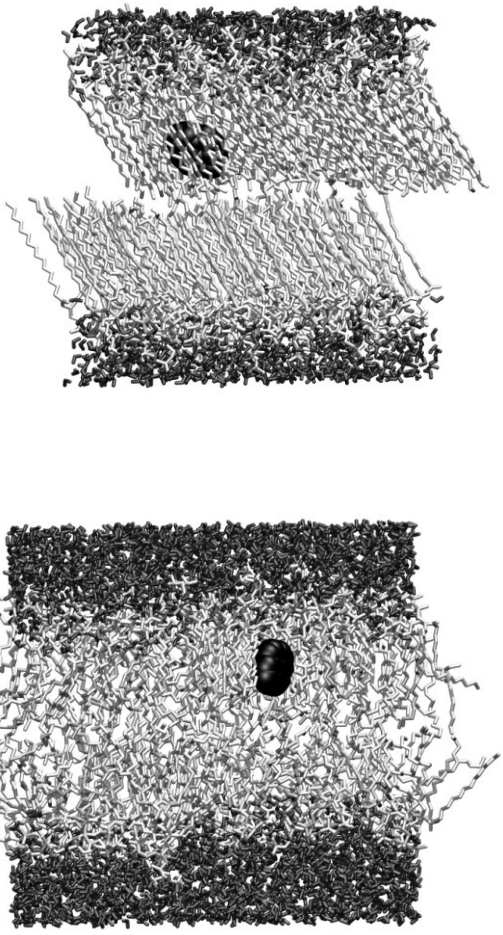
J. Čurdová [1], P. Čapková [1], J. Plášek[2], J. Repáková [3], I. Vattulainen[3]

[1]Department of Chemical Physics and Optics, Charles University, Ke Karlovu 3, 121 16 Prague 2, Czech Republic
[2]Institute of Physics, Faculty of Mathematics and Physics, Charles University, Ke Karlovu 5, 121 16 Prague 2, Czech Republic
[3]Laboratory of Physics and Helsinki Institute of Physics, Helsinki University of Technology, P.O.Box 1100, FI-02015 HUT, Finland

Most of the experimental studies of lipid membrane are based on the fluorescence technique.

Since membrane do not exhibit any fluorescence the fluorescent probes are used. One of the most common is pyrene. The aim of our study was to investigate influence of this pyrene probe to the structure and dynamics of dipalmytoilphosphatidylcholine (DPPC) bilayer by means of molecular dynamics simulation. Although, this DPPC bilayer has the main phase tranistion between gel and liquid crystalline phase at 314 K. So we had to make several simulations on different tempretatures and phases.

We have employed 20 ns and 50 ns molecular dynamics simulations of DPPC lipid membrane with different concentrations of pyrene fluorescent probe at temperatures below and above the main phase transition of DPPC. Molecular dynamics simulations shows that the pyrene prefers position in the hydrophobic acyl chains region close to the headgroup region of DPPC molecules, concretely between atoms C4 and C7. The most probable orientation of pyrene is approximately parallel with the bilayer normal but its orientation slightly dependents on the structure of the membrane. Further, pyrene affects ordering of close DPPC molecules. Although this effect with increasing distance from the probe the influence diminishes and at distance larger then 1.5 nm it is negligible. Finally, there was found the decrease of diffusion coefficient at lower temperatures and with additional probes in the DPPC membrane. The summary is that, the presence of pyrene in the membrane do not affect noticeably their properties and it is suitable for study of the lipid membrane.



a)

b)

Fig. 1. A snapshot of the lipid bilayers with pyrene fluorescent probe in a} gel and b) liquid crystalline phase.

P9

OPTICAL SPECTROSCOPY, MASS SPECTROMETRY AND NMR ARE ESSENTIAL TOOLS IN THE PRODUCTION OF SOLUBLE RECEPTORS OF NATURAL KILLER CELLS

K. Bezouška[1,2], D. Kavan[1,2], O. Vaněk[1], P. Pompach[1,2], L. Mihók[1,2], J. Bílý[1], P. Novák[2], K. Hofbauerová[2,3], V. Kopecký, Jr.[3], M. Nálezková[4], L. Žídek[4], V. Sklenář[4]

[1]Dept. of Biochemistry, Faculty of Science, Charles University, Hlavova 8, CZ-12840 Praha 2;
[2]Institute of Microbiology, Academy of Sciences of Czech Repub., Vídeňská 1083, CZ-14220 Praha 4;
[3]Institute of Physics, Faculty of Mathematics and Physics, Charles University Prague, Ke Karlovu 5, CZ-12116 Praha 2;
[4]National Centre for Biomolecular Research, Faculty of Science, Masaryk University in Brno, Kotlarska 2, 611 37 Brno, Czech Republic.
E-mail: bezouska@biomed.cas.cz

Natural killer (NK) cells represent an important lymphocyte subset critical in the immune response against viruses, certain microorganisms, and tumors. Effector functions of these cells are dependent on the balance between signals transmitted from both activating and inhibitory surface receptors [1]. While the inhibitory receptors interacting with MHC class I as ligand have been well characterized, much less is known about the activating receptors and their ligands [2,3]. In our laboratory we have been producing **soluble recombinant forms** of the two major activating receptors of NK cells, **CD69** and **NKR-P1.** Our production protocol includes bacterial expression of the extracellular portion of the above receptors. Precipitation into the inclusion bodies is followed by *in vitro* refolding and purification by a combination of ion exchange, hydrophobic, and gel permeation chromatographies. The purified proteins appeared to be **homogeneous on SDS polyacrylamide gel electrophoresis** displaying a notable shift towards higher mobility under the nonreducing conditions. They are refolded in the form of **noncovalent dimers** as revealed by gel filtration and cross-linking experiments. The verification of the proper folding after *in vitro* procedure is critical for all the subsequent protein applications. For the **initial assessment** of the refolding efficiency, we use a combination of the **UV, FT-IR** and **Raman spectroscopy**. UV spectroscopy serves as a good first test of the stability of the produced protein (thermal denaturation curves measured in varying chemical environments). Vibrational spectroscopy, on the other hand, is able to estimate the content of secondary structure elements (such as -helices, -sheets etc.) as well as structural details of certain amino acids such as cysteines. For the **final verification** of the folding status, **high resolution methods** that look onto proteins in the high magnetic fields are necessary. We use ion **Fourier transform-ion cyclotron resonance mass spectrometry** to look at the identity of the entire protein and the number of closed disulfide bonds. Measurement of the **1H – 15N HSQC spectra in a 600 MHz NMR spectrometer** using the uniformly ^{15}N labeled protein provides the definitive evidence for the proper folding and long-term stability of the protein preparations. The above measurements revealed that our preparations of soluble **rat and human CD69 protein** is stable for weeks / months at temperatures between 4 °C and 30 °C, while the short term stability up to 80 °C is worth mentioning [4]. Also, our most recent measurements of 1H – 15N HSQC spectra of the **rat NKR-P1A protein** points to the success of the currently employed refolding protocol. The use of NMR as well as protein crystallography and other methods for the ligand identification and verification experiments is under progress.

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P10

PREPARATION OF ANTENNARY OLIGOSACCHARIDE LIGANDS FROM OVOMUCOID AND THEIR BINDING TO CD69 RECEPTOR

M. Navrátil[1], P. Pompach[2], K. Bezouška[1,2]

[1]Department of Biochemistry, Faculty of Science, Charles University in Prague, Hlavova 8, CZ-12840 Prague
[2]Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-14220 Prague 2

Oligosaccharides form a very heterogeneous group of compounds. The ability of their monosaccharide units to bind to each other in many ways predicts them to be very variable in both structure and function. It is well known that saccharide structures play important role in immune system, from direct recognition of pathogenic saccharide structures to protein glycosylation, which can have also modulatory or self-protective function. One group of very interesting oligosaccharide compounds are branched antennary oligosaccharides which are present at the surface of tumour cells and are recognized by C-type lectin-like receptors of NK cells. Immune recognition is highly specific and only small difference in epitope structure may lead to opposite response, exact knowledge of this structure is therefore a necessity. Chemical synthesis [1] of antennary oligosaccharide structures is quite difficult. In contrast, there are glycoproteins that provide defined oligosaccharide structures in sufficient yields. Thus, the isolation of native structures from natural sources seems to be an appropriate way as well. In order to research for physiological ligands [2, 3] for C-type lectin-like receptors of NK cells we developed a relatively fast method for preparation of pure and well defined antennary oligosaccharides from hen egg white protein ovomucoid. This procedure involves isolation of ovomucoid by fractional precipitation, followed by its purification on HPLC, enzymatic deglycosylation of ovomucoid and finally purification and separation of cleaved oligosaccharides by HPLC. The purity and identity was verified by MALDI-TOF mass spectrometry. Biochemical binding and inhibition experiments with CD69 receptor revealed the highest affinity for the pentaantennary oligosaccharide. Oligosaccharides were used for structural studies by means of cocrystallisation with CD69, and examination of CD69-saccharide complexes by MS and NMR titration.

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