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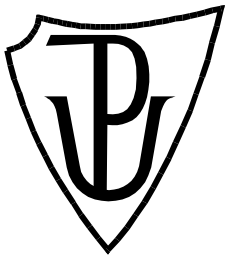
Faculty of Science

Department of Biochemistry



Analysis of post-translational modifications of proteins using mass spectrometry

Ph. D. Thesis

Vojtěch Franc

Olomouc 2014

Proclamation

"I hereby declare that the submitted thesis has been written by myself with the use of references. I performed the experiments on my own or with the assistance of my colleagues. Published data come from the following three scientific articles. Unpublished data and information are discussed in corresponding sections."

Paper I

Franc V., Šebela M., Řehulka P., Končitíková R., Lenobel R., Madzak C., Kopečný D. (2012) Analysis of N-glycosylation in maize cytokin oxidase/dehydrogenase 1 using a manual microgradient chromatographic separation coupled offline to MALDI-TOF/TOF mass spectrometry. *J. Proteomics*, **75**, 4027-4037.

Paper II

Franc V., Řehulka P., Medda R., Padiglia A., Floris G., Šebela M. (2013) Analysis of the glycosylation pattern of plant copper amine oxidases by MALDI-TOF/TOF MS coupled to a manual chromatographic separation of glycans and glycopeptides. *Electrophoresis*, **34**, 2357-2367.

Paper III

Franc V., Řehulka P, Raus M., Stulík J., Novák J., Renfrow M. B., Šebela M. (2013) Elucidating heterogeneity of IgA1 hinge-region O-glycosylation by use of MALDI-TOF/TOF mass spectrometry: role of cysteine alkylation during sample processing. *J. Proteomics*, **92**, 299-312.

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Bibliographical identification

First name and surname	Vojtěch Franc
Title	Mgr.
Type of thesis	Ph.D. thesis
Department	Department of Biochemistry
Supervisor	prof. Mgr. Marek Šebela, Dr.
Co-supervisor	RNDr. Pavel Řehulka, Ph.D.
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Abstract	The introduction of this Ph. D. thesis describes the role of mass spectrometry (MS) in biology and the significance of proteomic in analysis of post-translational modifications (PTMs) of proteins. Further, the thesis deals with post-translational modifications of proteins, especially *N*- and *O*-glycosylation. It describes the importance of *N*- and *O*-glycans and some strategies of their analysis by MS. The experimental part is divided into three sections: 1) An overview of methods, 2) Analysis of *N*-glycosylation in plant proteins, and 3) Analysis of *O*-glycosylation in the hinge-region (HR) of polymeric myeloma immunoglobuline alpha 1 (pIgA1). The first part describes a strategy of analytical workflow and deals with a majority of methods that have been used (especially a microgradient device). In the second part, studies on several plant glycoproteins – cytokinin oxidase/dehydrogenase (CKO; EC 1.5.99.12) and copper-containing amine oxidases (CAOs; EC 1.4.3.22, formely EC 1.4.3.6) are introduced. An enzymatic deglycosylation of CKO and CAOs by endoglycosidase H under denaturing conditions combined with their proteolytic digestion by trypsin was carried out in order to analyze both *N*-glycans and "trimmed" *N*-glycopeptides with a residual *N*-acetylglucosamine attached at the originally occupied *N*-glycosylation sites. The released *N*-glycans were subjected to a manual chromatographic purification followed by MALDI-TOF/TOF MS and MS/MS (matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight mass spectrometry and tandem mass spectrometry). Tryptic *N*-glycopeptides (in the case of CAOs also other proteases were used) were either measured directly with MALDI-TOF MS or separated by use of revesed-phase liquid chromatography (RPLC) and subsequently analyzed by MALDI-TOF/TOF MS and MS/MS. The RPLC separation by use of a simple microgradient device provided simplification of a glycopeptide mixture and resulted in an improved analysis of glycosylation modifications. Recombinant maize CKO isoenzyme 1 (ZmCKO1) expressed in *Yarrowia lipolytica* contained 9 potential *N*-glycosylation sites. Glycopeptide sequencing confirmed *N*-glycosylation at Asn52, 63, 134, 294, 323 and 338. Interestingly, Asn338 was the sole site to carry large glycan chains exceeding 25 mannose residues. CAO enzymes, pea and lentil seedling amine oxidases (PSAO

and LSAO, respectively) were purified as native proteins from their natural sources. MS and MS/MS data clearly demonstrated binding of paucimannose and hybrid *N*-glycan structures at Asn558.

The third part describes analysis of a pIgA1 protein simulating IgA from IgA nephropathy (IgAN) patients. The primary aim was to identify *O*-glycopeptides and to determine the localization of the corresponding *O*-glycans in the HR of IgA1. For this purpose, the analyzed sample of the pIgA1 was first separated by use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The resulting electropherogram showed two protein bands with apparent molecular masses of 63 kDa and 28 kDa that correspond to heavy and light chain of IgA1. Because the peptide sequence of the HR contained three cysteine residues, it was also necessary to optimize the reduction/alkylation procedure of the pIgA1 sample with the respect to the SDS-PAGE separation. This resulted in removal of undesired peptide modifications and substantially improved signal response of analyzed *O*-glycopeptides. The gel band containing separated heavy chain of IgA1 was excised from the gel and in-gel digested with trypsin. Then the peptide mixture was separated by the microgradient device and further analyzed by MALDI-TOF/TOF MS and MS/MS. One sample fraction contained a mixture of various *O*-glycoforms of the HR tryptic peptide. Manual and software interpretation of MS/MS spectra of these *O*-glycopeptides provided unambiguous localization of all *O*-glycosylation sites in 15 most abundant glycoforms including the glycoforms deficient in galactose (Gal). These results represent the first direct site assignment of multiple *O*-glycosylation with complex heterogeneity of the pIgA1 HR by MALDI-TOF/TOF MS and MS/MS.

Keywords	glycoproteomics, post-translational modification, glycosylation, mass spectrometry, microgradient separation
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Jméno a příjmení	Vojtěch Franc
Titul	Mgr.
Druh práce	Disertační práce
Pracoviště	Katedra biochemie
Školitel	prof. Mgr. Marek Šebela, Dr.
Odborný konzultant	RNDr. Pavel Řehulka, Ph.D.
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Abstrakt

V úvodu této disertační práce je popsána pozice hmotnostní spektrometrie (MS) v biologii a význam proteomiky v analýze posttranslačních modifikací (PTM) proteinů. Dále pojednává o posttranslačních modifikacích proteinů, obzvláště pak o *N*- a *O*-glykosylacích. Popsána je důležitost *N*- a *O*-glykanů a některé strategie jejich MS analýzy. Experimentální část je rozdělana na tři části: 1) Přehled metod, 2) Analýza *N*-glykosylace v rostlinných proteinech, a 3) Analýza *O*-glykosylace v pantové oblasti (HR) polymerního myelomového imunoglobulinu alpha 1 (pIgA1).

První část popisuje strategii analytického pracovního postupu a zabývá se hlavními použitými metodami (obzvláště mikrogradientovým zařízením).

Druhá část se zabývá rostlinnými glykoproteiny – cytokinin oxidasou/dehydrogenasou (CKO; EC 1.5.99.12) a měď-obsahujícími aminoxidasami (CAO; EC 1.4.3.22, dříve EC 1.4.3.6). Za účelem analýzy *N*-glykanů a deglykosylovaných *N*-glykopeptidů obsahujících residuální *N*-acetylglukosamin, který byl vázán na původně obsazených *N*-glykosylačních místech, byla provedena enzymová deglykosylace CKO a CAO endoglykosidasou H za denaturujících podmínek kombinovaná s proteolytickým štěpením trypsinem. Uvolněné *N*-glykany byly podrobeny manuální chromatografické purifikaci následované MALDI-TOF/TOF MS a MS/MS analýzou. Tryptické *N*-glykopeptidy (v případě CAO byly použity i jiné proteasy) byly analyzovány přímo MALDI-TOF MS nebo separovány použitím kapalinové chromatografie s obrácenou fází (RPLC) a následně analyzovány MALDI-TOF/TOF MS a MS/MS. RPLC separace použitím jednoduchého mikrogradientového zařízení poskytla zjednodušení glykopeptidové směsi a vyústila ve zlepšenou analýzu glykosylačních modifikací. Rekombinantní kukuřičný CKO isoenzym 1 (ZmCKO1) exprimovaný v *Yarrowia lipolytica* obsahoval 9 potenciálních *N*-glykosylačních míst. Glykopeptidová sekvenční analýza potvrdila *N*-glykosylaci na Asn52, 63, 134, 294, 323 a 338. Zajímavé je, že Asn338 jako jediný mezi glykosylačními místy nesl velké glykanové řetězce přesahující až 25 manosových zbytků. CAO enzymy, aminoxidasy ze semenáčků hrachu a čočky (PSAO a LSAO), byly purifikovány jako nativní proteiny z jejich přírodních zdrojů. MS a MS/MS data jasně demonstrovala

vázání paucimanosových a hybridních *N*-glykanových struktur na Asn558.

Ve třetí části se píše o analýze proteinu pIgA1 simulujícího IgA z pacientů trpících IgA nefropatií (IgAN). Primárním cílem byla identifikace *O*-glykopeptidů a lokalizace odpovídajících *O*-glykanů v pantové oblasti (HR) IgA1. Za tímto účelem byl analyzovaný vzorek pIgA1 nejprve separován použitím SDS-PAGE. Vysledný elektroferogram ukázal dva proteinové pásy s molekulovými hmotnosnostmi 63 kDa a 28 kDa, které odpovídaly těžkému a lehkému řetězci IgA1. Protože peptidová sekvence HR obsahovala tři cysteinová residua, bylo nezbytné optimalizovat podmínky redukce/alkylace vzorku pIgA1 s ohledem na separaci SDS-PAGE. To vyústilo v odstranění nežádoucích modifikací peptidů a zlepšilo odezvu signálu analyzovaných *O*-glykopeptidů. Pás se separovaným těžkým řetězecem IgA1 byl vyříznut z gelu a protein v něm štěpen trypsinem. Získaná peptidová směs separovaná mikrogradientovým zařízením byla dále analyzována MALDI-TOF/TOF MS a MS/MS. Jedna frakce ze vzorku obsahovala směs různých *O*-glykoforem HR tryptického peptidu. Manuální a softwarová interpretace MS/MS spekter těchto *O*-glykopeptidů poskytla jednoznačnou lokalizaci všech *O*-glykosylačních míst v 15 nejvíce zastoupených glykoformách zahrnujících i glykoformy deficientní na galaktosu (Gal). Tyto výsledky představují první přímé stanovení míst mnohočetných *O*-glykosylací s komplexní heterogenitou v pIgA1 HR použitím MALDI-TOF/TOF MS a MS/MS.

Klíčová slova: glykoproteomika, posttranslační modifikace, glykosylace, hmotnostní spektrometrie, mikrogradientová separace

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Contents

1. Introduction

1.1 Mass spectrometry-based biology

In 1913, J. J. Thomson published and edited a monograph called “Rays of positive electricity and their application to chemical analyses”. He summarized there the experiments on “Positive Rays” which had been done at the Cavendish Laboratory at the University of Cambridge during the last seven years. In the preface of the book, Thomson says: “I have described at some length the application of Positive Rays to chemical analysis; one of the main reasons for writing this book was the hope that it might induce others, and especially chemists, to try this method for analysis. I feel sure that there are many problems in Chemistry which could be solved with far greater ease by this than by any other method.” (Thomson, 1913). The method, which Thomson so modestly suggested for the use, was “mass spectrometry”; and he would probably never imagine, how big truth was hidden in his words. MS has found application not only in chemistry but also in medicine, biochemistry, biology and many other various fields.

1.1.1 Soft ionization techniques – MALDI and ESI

Despite the fact that MS has its roots back to the early years of the last century, the way to biological application of this method has been quite long. The breakthrough came with the development of “soft ionization” techniques (MALDI and ESI) which allowed transfer of large and polar biomolecules into the gas phase and their ionization. Subsequently, these techniques became basis for the increasingly powerful instrumentation that started to be available in the last years.

MALDI technique was developed by Karas and Hillenkamp in the late 1980s (Karas & Hillenkamp, 1988). After development of MALDI, Tanaka reported so called “soft laser desorption” (SLD), which has induced wide use of MALDI for ionization of protein molecules (Tanaka *et al*, 1988). Although the precise nature of the ionization process in MALDI is still largely unknown, the basic principles can be described as follows: a large excess of matrix material is co-crystallized with analyte molecules. Next, the creation of gas-phase ions is achieved by nanosecond laser pulses focused to the solid mixture of matrix and analyte. After irradiation of the crystals, the photon energy deposited into the matrix converts to thermal energy, causing rapid evaporation of matrix and analyte (Yates, 2001; Mann *et al.*, 2001). These short bursts of ions produced in the vacuum are usually analyzed with TOF MS, nevertheless MALDI can be coupled also with other types of analyzers like for example Q/TOF (Shevchenko *et al*. 2000) or IT (ion trap)/TOF (Liu, 2012).

“Electrospray Wings for Molecular Elephants” – that is how John B. Fenn called his “Nobel Lecture” (December, 2002) related to the second key ionization technique for use of

MS in biology (Fenn *et al*. 1989). To form electrospray (ESI), an analyte dissolved in liquid phase is sprayed from the tip of a capillary at a high voltage (0.5-4 kV). Small, micrometer-sized droplets are created and the solvent contained in the droplets is rapidly evaporated. This causes their shrinking and subsequent increasing charge per unit volume. The gradual accumulation of charge leads to desorption of ions from the surface of the droplet. Next, the created ions are transferred into the mass spectrometer with high efficiency for analysis. One of the greatest benefits of ESI is the creation of a robust interface between liquid separation techniques and the mass spectrometer. This allows a comprehensive chromatographic or electrophoretic separation of complex peptide mixtures and their subsequent analysis in mass spectrometer (Yates, 2001).

1.1.2 MS for biologists

Proteomics is becoming a central field in functional genomics and the core method for large-scale analyses of the function of genes is MS (Pandey & Mann, 2000). In the field of biotechnology, MS is the major tool for characterization and quality control of recombinant proteins. It is also preferred method for protein identification. Because mass spectrometer measures the molecular weight (to be more exact, it measures *m/z* rather than the mass), it enables the identification and characterization of PTMs or any covalent modification altering the mass of a protein (Mann *et al*., 2001). The interesting fact is that MS-based proteomics (or biology) has stopped to be privileged field of MS laboratories and more and more biologists solve their biological questions and problems by use of mass spectrometers. This trend leads to rapid expanding of biological MS not just in terms of the methods and instruments but also the biological questions.

Analyses of protein-protein interactions, protein-small molecules interactions, compartmentalization, study of catalytic activity or the signaling pathways belong among the most interesting applications of MS (Baker, 2010). Combining MS with other structural methods and molecular biology methods provides a very powerful tool for the study of life on molecular level. For example, a genome-scale analysis of protein complexes in the genome-reduced bacterium *Mycoplasma pneumoniae* provided a map of the minimal cellular machinery required for life. The power of the MS lies in the ability to reveal protein interactions which have not been predicted or expected (Kühner *et al*. 2009). Divergent data sets were also integrated in order to find a protein that allows the innate immune system to sense and respond to pathogens. In this study, an orthogonal genomic-proteomic screen combining data from electron microscopy, tomography and MS led to identification of interferon-inducible protein (AIM2 – “absent in melanoma 2”) as a cytoplasmatic DNA sensor for the inflammasome (Bürckstümmer *et al*., 2009).

Mass spectrometers are faster, more sensitive and easier to use. These factors together with better protein purification and separation techniques are responsible for the shift toward quantitative proteomics. An effort to combine quantitative MS with other structural biology techniques brings new insights into the compartmentalization of protein complexes. So called “visual proteomics” approach was described by Aebersold and his colleagues. Cryoelectron tomography, a three-dimensional imaging technique together with quantitative MS allowed to count and localize protein complexes in the human pathogen *Leptospira interrogans* (Beck *et al*. 2009).

1.2 Significance of proteomic in study of post-translational modifications

In 2003, one of the greatest and the most ambitious research projects in the whole history of mankind called “Human Genom Project”, which has launched a new era in biological sciences, was finished (Schmutz *et al*., 2004). However, the knowledge of amino acid sequence alone, which determines the primary structure of protein, is by far insufficient to explain its variable biological functions. After proteosynthesis on ribosomes proteins are covalently processed by proteolytic cleavage or by addition of a modifying group to one or more amino acids. Generally, these events are called PTMs which change the properties of proteins. PTMs of protein can determine its activity state, localization, turnover, and interactions with other proteins (Seo & Lee, 2004; Mann & Jensen, 2003).

The understanding of relationship between PTMs and alternation of protein functions (“post-translomics”) is another great challenge for biological sciences. Proteomics or more specifically proteomics methods including MS have become very successful and effective tool for identification of proteins in complexes and organelles (Mann & Jensen, 2003). The number of proteins credibly identified in a shotgun study in the year 2000 was maybe 100 up to 300. Now, the state of the art would be 4000 up to 5000 identified proteins in one experiment. (Mitchell, 2010). Once proteins are identified, the next question is whether or not proteins are post-translationally modified and how. There are several reasons why analysis of PTMs is more complicated than the identification of proteins. Firstly, for an identification of proteins, it is sufficient to sequence and map only a few peptides while a mapping of PTMs requires the complete sequence coverage of a protein. Secondly, since the covalent bond between the PTM and amino acid side chain is typically labile, it is often difficult to maintain the peptide in its modified state during sample preparation and subsequent ionization in MS. Thirdly, the protein modifications *in vivo* occur only in a small fraction of total proteins (less than 1%). Furthermore, the analyzed samples are usually heterogeneous mixtures of variably modified and unmodified proteins and PTMs are frequently transient in a dynamic homeostasis of nature. For these reasons, new very sensitive and in the same time robust methodologies are required for a detection of low

abundant molecules (less than 5-10 fmol) and for a thorough mapping of all cellular endogenous protein modifications. Although the present proteomic technology is able to detect only simple modifications in large amounts of modified samples, its potential and significance for understanding of PTMs is enormous (Seo & Lee, 2004).

Tab. 1

Some common and important PTMs (adopted with small alternations from Mann & Jensen, 2003)

PTM type	**Δ Mr[a] (Da)**	**Modified amino acid residue**	**Stability[b]**	**Function and notes**
Phosphorylation	+80	Tyr Ser Thr	+++ +/++ +/++	Reversible, activation/inactivation of enzyme activity, modulation of molecular interactions, signaling
Acetylation	+42	S, K	+++	Protein stability, protection of *N*-term. Regulation of protein-DNA interactions (histones)
Methylation	+14	K	+++	Regulation of gene expression
Acylation, fatty acid modification				Cellular localization and targeting signals, membrane tethering, mediator of protein-protein interactions
Farnesyl	+204	C	+++	
Myristoyl	+210	G, K	+++	
Palmitoyl	+238	C (S, T, K)	+/++	
etc.				
Glycosylation				
N-glycosylation	>800	N	+/++	Excreted proteins, cell-cell recognition/signaling, reversible, regulatory functions
O-glycosylation	203, >800	S, T	+/++	
GPI anchor	>1000	C-term	++	Glycosylphosphatidylinositol (GPI) anchor. Membrane tethering of enzymes and receptors, mainly to outer leaflet of plasma membrane
Hydroxyproline	+16	P	+++	Protein stability, blocked *N*-term.
Sulfation	+80	Tyr	+	Modulator of protein-protein and receptor-ligand interactions

Disulfide bond formation	-2	C	++	Intra- and intermolecular cross-link, protein stability
Deamidation	+1	N, Q	+++	Possible regulator of protein-ligand and protein-protein interactions, also a common chemical artifact
Pyroglutamic acid	-17	Q	+++	Protein stability, blocked *N*-term.
Ubiquitination	>1000	K	+/++	Destruction signal. After tryptic digestion, ubiquitination site is modified with the Gly-Gly dipeptide
Nitration	+45	Y	+/++	Oxidative damage during inflammation

[a]A more comprehensive list of PTMs Δmass values can be found at: http://www.abrf.org/index.cfm/dm.home

[b]Stability: + labile in MS/MS, ++ moderately stable, +++ stable.

1.3 Glycosylation – uncrowned queen among PTMs

Glycomics and glycoproteomics are the study of a cell, tissue, or organism´s glycan and glycoprotein content at any point in time (http://ncgg.indiana.edu/). The significance and outstanding position of protein glycosylation among other PTMs can be expressed by following citations which has been randomly chosen from various research papers and reviews related to PTMs or glycosylation:

„Protein glycosylation is more abundant and structurally diverse than all other types of post-translational modifications combined. Protein-bound saccharides range from dynamic monosaccharides on nuclear and cytoplasmatic proteins, to enormously complex recognition molecules on extracellular *N*- or *O*-linked glycoproteins or proteoglycans. Recent elucidation of a few of the myriad functions of these saccharides has finally opened a crack in the door to one the last great frontiers of biochemistry" (Hart, 1992).

"Glycobiology research has attracted increasing attention because glycosylation is the most complex and most frequently occurring post-translational modification. Similar to the developments in genomics and proteomics, high-throughput glycomics projects to decipher the role of carbohydrates in health and disease are emerging." (von der Lieth, 2009)

"Glycosylation is one of the most important and common forms of protein post-translational modification that is involved in many physiological functions and biological pathways" (Pan *et al*., 2011).

In the special issue of *Science* dedicated to glycobiology (March 2001 edition) editors compared chemistry and biology of carbohydrates to a "Cinderella field" of research.

It was described as “an area that involves much work but, alas, does not get to show off at the ball with her cousins, the genomes and proteins” (Hurtley *et al*., 2001). More than 10 years later after editing of this essential issue (Fig. 1), carbohydrates related research has transformed from a “shy Cinderella” to a “proud, self-confidence queen” who has taken the position of one of the most progressive and ambitious fields of interest in biomolecular and biomedical research.

Fig. 1

The cover of a special issue of *Science* dedicated to glycobiology science has become very popular throughout the whole scientific world. The cell surface landscape is richly decorated with glycans anchored to proteins or lipids within the plasma membrane. Cell surface glycans mediate the interactions of cells with each other and with extracellular matrix components. The important roles that carbohydrates play in biology and medicine have stimulated a rapid expansion of the field of glycobiology. Illustration: Cameron Slayden

https://www.sciencemag.org/content/291/5512.cover-expansion

2. Classification and structure of glycans

Glycosylation is classified according to the type of the atom via which glycans are attached to a protein. The first and the most common type is the *N*-glycosylation and the

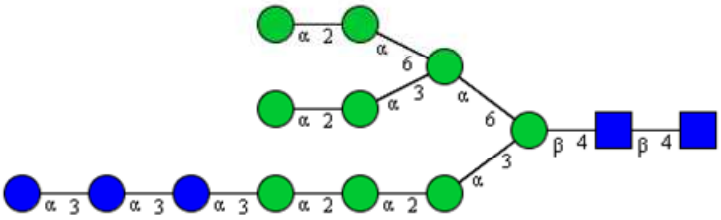
second is the *O*-glycosylation. The third major class is the glycosylation of lipids (Herget *et al*., 2009) which this thesis will not discuss. In addition to this, the new type of glycosylation has recently been discovered in glycopeptide bacteriocins – S-glycosylation at Cys residues (Stepper *et al*., 2011).

2.1 *N*-glycosylation

All eukaryotic cells express glycoproteins which covalently attach glycans via the amide nitrogen of a specific asparagine (Asn). *N*-glycans are linked to a protein at locations having the amino acid sequence Asn-X-Ser/Thr (called "sequon"; where X is any amino acid except Pro; Marshall, 1972). During the recent years, some examples of glycosylation of Asn residues not in sequons have been reportet. It has been several times identified glycosylation at Asn in the sequence Asn-X-Cys (Vance *et al*., 1997; Miletich & Broze, 1990). In addition, large-scale discovery project for identification of *N*-glycosylation sites in mouse glycoproteins has revealed 20 glycosylated Asn in the sequence Asn-X-Val (Zielinska *et al*., 2010).

Glycan intermediates, which are supposed to be incorporated into the structure of a protein, are synthetized in a controlled mechanism. This process is mediated by a multi-enzyme complex oligosaccharyltransferase (OST; Kaplan *et al*., 1987). The OST catalyzes the transfer of the glycan with composition $Glc_3Man_9GlcNAc_2$ (Fig. 2a) from an activated dolichol pyrophosphate precursor, to the newly synthetized polypeptide chain. This intermediate glycan is subsequently processed in accordance with the correct protein folding (Helenius & Aebi. 2001). It was proposed that during transportation of the glycan to the Asn residue, the hydroxy amino acid (Ser/Thr) in the sequon serves as a hydrogen donor for the formation of the hydrogen bond which is essential for the function of the OST enzyme (Imperiali & Hendrickson, 1995). However, not all sequons presented in a protein are glycosylated. This means, that the sequon is necessary for glycosylation, but its presence itself is not conditional criterion for the glycan presence on particular site (Apweiler *et al*., 1999). Moreover, apparent glycosylation of Asn-X-Val sequons could not be explained by the proposed mechanism. The high-resolution 3D crystal structure of the bacterial OST enzyme provided a structural basis for the requirement of a glycosylation sequon (Lizak *et al*., 2011). The co-crystalized structure of OST with an acceptor peptide clearly showed that Thr in the sequon was too far away from the Asn to be directly involved in catalysis. Instead, the role of the glycosylation sequon is to increase the binding affinity of Asn to the active site of OST. Such interactions increasing the binding affinity are restricted in the presence of Pro next to the Asn (+1 and -1 position) which explained the structural requirement that Pro cannot be present at these positions at glycosylation sites.

(a)



(b)

Symbol	Abbreviation	Monosaccharide	Symbol	Abbreviation	Monosaccharide
	Gal	Galactose		Man	Mannose
	GalNAc	N-Acetylgalactosamine		ManNAc	N-Acetylmannosamine
	GalN	Galactosamine		ManN	Mannosamine
	GalA	Galacturonic acid		ManA	Mannurononic acid
	Glc	Glucose		Kdn	2-keto -3-deoxynonic acid
	GlcNAc	N-Acetylglucosamine		Neu5Ac	N-Acetylneuraminic acid
	GlcN	Glucosamine		Xyl	Xylose
	GlcA	Glucuronic acid		IdoA	Iduronic acid
	Fuc	Fucose		Neu5Gc	N-Glycolylneuraminic acid

Fig. 2

(a) Structure of a tetradecasaccharide $Glc_3Man_9GlcNAc_2$ which is sometimes also reffered to as the precursor of *N*-glycans. Glycosidic linkages are indicated by connecting lines between monosaccharide units. Binding position and anomeric state are labeled for each linkage. (b) Symbols assigning glycan structures which have been approved by the "Consortium for Functional Glycomics" (CFG). Circles indicate hexoses, squares *N*-acetylhexosamines, diagonally divided squares hexosamines and diamonds acidic sugars. Fucose and iduronic acids are in the *L*-configuration, all other basic monosaccharides are in the *D*-configuration (www.functionalglycomics.org).

-reconstructed from Herget *et al*., 2009; by means of software Glycoworkbench (Ceroni *et al*., 2008).

With a few exceptions (Spiro, 2002) all *N*-glycans share a common structural motif $Man_3GlcNAc_2$ (Fig. 3a). From this "core" structure, another three topological classes are derived. High-mannose type (Fig. 3b) arises if only mannose residues are attached to the core. Complex types usually do not contain additional mannoses, but rather they occur with directly linked GalNAc to the two branches of the *N*-glycan core. Complex types may be galactosylated, fucosylated and sialylated (Fig. 3c, e). The last common type of mammal *N*-glycosylation is a hybrid type, which contains both, high-mannose and complex motifs (Fig.

3d). Further empirical structural diversities of *N*-glycans include fucosylation and a connection of different antennas (Fig. 3e, f; Dennis *et al*., 1999).

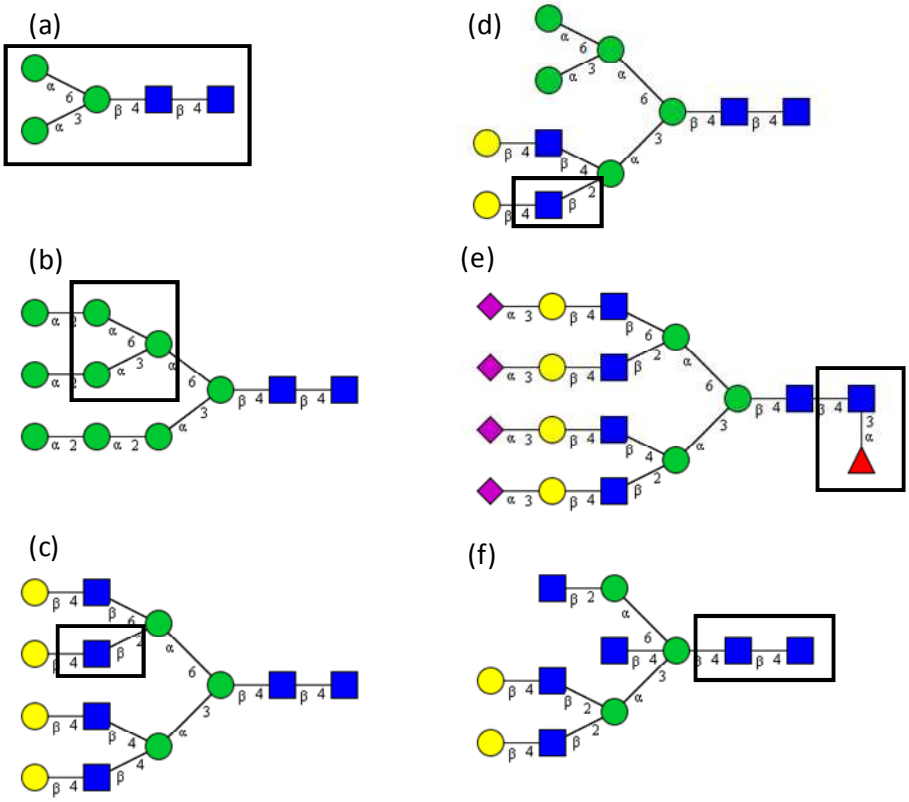


Fig. 3

Common types of *N*-glycans. The boxes indicate a typical structural features leading to the appropriate classification of glycans: (a) the basic core characteristic for all *N*-glycans; (b) high-mannose types are formed by modifying of the initial structure $Glc_3Man_9GlcNAc_2$ (see Fig. 2a) which is transported to the Asn residue of glycoprotein; (c) *N*-glycan core is further expended by connection of the GlnNAc and Gal residues thereby it gives to form a complex type of *N*-glycans; (d) hybrid type contains both, high-mannose type and complex type structural features; (e) *N*-glycan with fours antennas and with α(1-3)-*L*-fucose attached to the core; (f) bisected *N*-glycan. Imaginary bisection of the structure is defined by connection of the β(1-4) *D*-GlcNAc to the central mannose forming the core.

-reconstructed from Herget *et al*., 2009 by means of software Glycoworkbench (Ceroni *et al*., 2008).

2.2 *O*-glycosylation

Generally, we talk about *O*-glycosylation of a protein when a mono- or glycan is attached to the amino acid residue with a functional hydroxyl group. Specifically, these are Ser, Thr or in a lesser extent Tyr, hydroxyproline and hydroxylysine. Unlike *N*-glycosylation where the only single enzyme localized in endoplasmatic reticulum (ER) is responsible for

the initial step (transmission of precursor glycan to the Asn) an attachment of a monosaccharide to the amino acid in the case of *O*-glycosylation may catalyze several different glycosyltransferases (GT). It may be also the reason, why so far, no characteristic sequence motif comparable to the *N*-glycosylation sequon has been identified (Wopereis *et al*., 2006). The complexity of determination of such a sequon is illustrated by the following fact: even if every single enzyme which is involved in the initial step of *O*-glycosylation preferentially recognizes any particular sequence motif, the result would be only ambiguous, chaotic data due to possible overlapping of sequence motifs for variable GT (Herget *et al*., 2009).

For historical reasons, the *O*-glycosylation is understood as an attachment of the GalNAc in α-configuration to Ser or Thr residue by the activity of the enzyme family GalNAc transferases in the secretory pathway during a maturation of a protein in the ER and the Golgi apparatus (GA). From this perspective, *O*-glycans may be defined by dividing into eight sub-classes according to the incidence of common structural motifs at the reducing end, and it is only matter of so-called mucin type *O*-glycosylation (Fig. 4; Herget *et al*., 2009). If we use the generall definition of *O*-glycosylation, it can be classified into seven main classes (Tab.2) according to the first carbohydrate attached to Ser, Thr or hydroxylysine (Wopereis *et al*., 2006).

Fig. 4

Mucin-type *O*-glycans. Eight cores of *O*-linked glycans of the mucin type have always common the *D*-GalNAc and differ from each other according common structural elements at the reducing termini.

-reconstructed from Herget *et al*., 2009 by means of software Glycoworkbench (Ceroni *et al*., 2008).

Tab. 2

Types of human *O*-glycans		
Type of *O*-glycan	**Structure and peptide linkage**	**Glycoprotein**
Mucin type	(R)-GalNAcα1-Ser/Thr	Secreted + plasma membrane protein
GAG	(R)-GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-Ser	Proteoglycans
O-GlcNAc	GlcNAcβ1-Ser/Thr	Nuclear and cytoplasmatic
O-Gal	Glcα1-2Galβ1-O-Lys	Collagens
O-Man	NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-Ser/Thr	α-Dystroglycan
O-Glc	Xylα1-3(Xylα1-3)Glcβ1-Ser	EGF protein domains
O-Fuc	NeuAcα2-6Galβ(1-4GlcNAcβ)1-3Fucα1-Ser/Thr	EGF protein domains
	Glcβ1-3Fucα1-Ser/Thr	TSR repeats

Seven main classes of *O*-glycosylation (adopted from Wopereis, *et al*., 2006)
(EGF – epidermal grow factor; TSR – thrombospondin-1 type repeats)

3. Function of protein glycosylation

Over a decade ago, it was estimated that up to 50% of human proteins are glycosylated (Apweiler *et al*., 1999). A more recent statistical study of the SWISS-PROT database suggested that 20% are glycoproteins. The study also revealed that *N*-linked glycosylation was the third most abundant and *O*-linked glycosylation the seventh most abundant protein modification (Khoury *et al*., 2011). Even though these studies are somehow in a contradiction, both of them bring clear message that the number of glycosylated proteins is enormous. From this point of view there must be very good reason why cells produce glycosylated proteins. Thus determine a precise biological and physiological role of glycosylation, generally represents a very complex problem and to answer the simple question, "What is the function of glycosylation?", is in many particular cases unclear and covered by mystery. One of the most distinguished experts in the field of glycobiology professor Ajit Varki from University of California, published in 1993 a review entitled "Biological roles of glycans: all of the theories are correct" (Varki, 1993). Although this review is not focused only on the protein glycosylation but also on other glycoconjugates, for a description of complexity and variability of the biological function of protein glycosylation is the title of the review more than cogent.

Although the most common function for glycans is to contribute to the stability of proteins to which they are attached, characteristic glycoforms are involved in recognition events (Rudd *et al*., 2001). Essential and more specific biological roles of glycans are very

often mediated by uncommon glycan sequences, extraordinary presentations of common terminal sequences, or by further modifications of glycans themselves. It seems that common features of variable functions of glycans are generally a “modulation” of protein structure and function, and “specific recognition” events (Hurtley *et al*., 2001; Varki, 1993).

If one starts to think about a function of glycosylation, it can result in another very basic but interesting questions. The cellular machinery which is involved in the synthesis and modulation of glycosylation is extremely complex and it by far exceeds exigencies for the synthesis and processing other covalent modifications of proteins. Also the size of glycosylation is much bigger than other modifications (Helenius & Aebi, 2001). So why do cells need such a sophisticated and costly system? Why are so many proteins in a eukaryotic cell glycosylated? Why do *N*-linked glycans undergo so many changes during glycoprotein maturation? It is important to realize that the primary structure of proteins is defined by their amino acid sequences and these polypeptide chains are naturally very limited in possibilities to generate branched structures. Addition of *N*- or *O*-glycan chains can provide a way to overcome this limitation.

4. Analysis of protein glycosylation by mass spectrometry

4.1 Limitations in analysis of glycosylation

The process of glycan biosynthesis is not controlled by a template. The synthesis of complex glycans involves many different enzymes which are characteristic by a defined specificity. Thus there are no biological amplification methods in the style of a polymerase chain reaction (PCR) as it is in the case of DNA. In practice, this means that glycans have to be analyzed in their physiological concentrations. From this perspective, the main problem is to develop highly sensitive analytical methods which can detect very small quantities of a sample.

The second major problem is the complexity of glycan structures. Every single pair of monosaccharide residues can be bound to each other in several ways and one residue may be associated with three or four other (branched structures). Information content which is potentially encoded by glycan chains is also enormous. Four nucleotides in DNA can be combined into 256 possible tetramers (4^4), four amino acids provide 160 000 possible tetramers (20^4). However, the structural variability of monosaccharide building unites found in nature is much greater than information encoding biological macromolecules and one can hypothetically create more than 15 million tetramer structures. Although glycans have potentially such a high capacity to store biological information, only a small part of it is truly utilized. Analysis of the database KEGG in 2004 (Kanehisa *et al*., 2004), which contains 4107 unique glycan structures (Kawano *et al*., 2005) comprising mostly 9 monosaccharides

(glucose, galactose, mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose, xylose, glucuronic acid and sialic acid), showed that only 302 (54 %) of 558 (9 monosaccharides, 2 anomers, 31 substitution possibilities) theoretically possible disaccharides are presented in this database. In addition, there are an enormous number of theoretically possible combinations of reaction schemes but only 2178 of these options really exist in the database. This statistical analysis indicates that the structural diversity of glycans is really huge, but combinating of reaction schemes which actually exist in the cellular environment is limited by the ability of enzymes involved in the biosynthesis of glycans to build and to modify their structure (von der Lieth, 2009).

Although eukaryotic cell has the same glycosylation machinery for all proteins, most of glycoproteins are created with characteristic glycosylation patterns and heterogeneous population of glycans in every single glycosylation site – glycoforms (Rudd & Dwek, 1997; Chalabi *et al*., 2006; Arnold *et al*., 2007). Glycoproteins generally exhibit macro- and microheterogenity. Macroheterogeneity (Fig. 5a), also termed as a "variable site occupancy", refers to a variability in the location and number of glycan attachments. On the other hand, microheterogeneity (Fig. 5b), also termed as a "site microheterogeneity", refers to a variability in the glycan structures at specific glycosylation sites. This heterogeneity leads to structural diversity of one specific glycoprotein, and thus to potential different functions of its glycoforms. Usually, more than 10 different glycans are observed in one glycosylation site but even up to 100 glycoforms were reported is some cases (Rudd & Dwek, 1997).

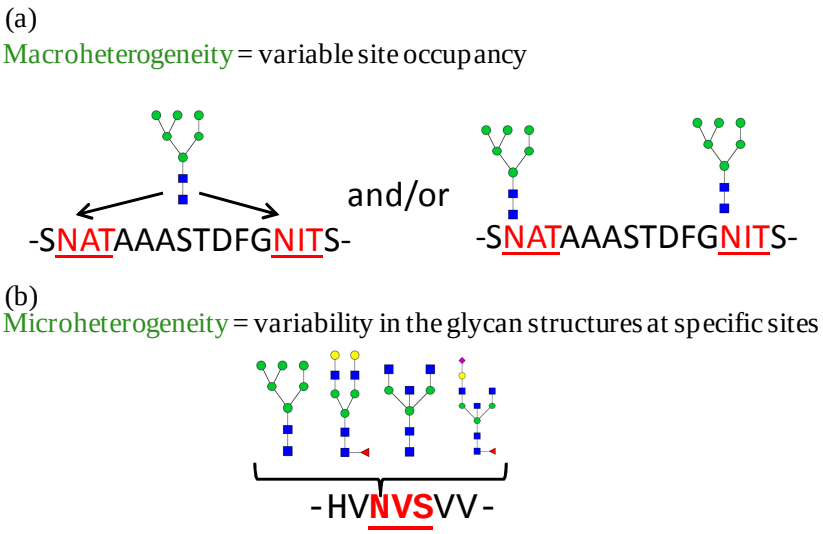


Fig. 5

Heterogeneity of glycoproteins.

Non-stoichiometric modifications and variety of chemical substitutions, which require development of completely new concepts of analysis, are other unique features of

complex glycans. An example could be statistical occurrence of suphate groups on specific positions in glycosaminoglycans as are heparin or heparan suphate (Coombe & Kett, 2005; Capila & Linhardt, 2002).

Glycoproteins participate in many aspects of cell biology. They are involved in recognition of pathogens, cellular transport, endocytosis and modulation of cellular signalization (Collins & Paulson, 2004). Assignment of the biological function of glycans in recognition events is complicated because individual glycans show very weak interactions with protein surface (Herrmann *et al*., 1997).

For these reasons, detailed analysis of glycans including all structure features is a very complex task. Procedures are usually time-consuming and a big amount of sample is required (in the order of micrograms). Very often such methods are used which are not able to detect all structural details of glycans. However, modern analytical methods can clarify most of structural features in the range of concentrations required in glycoproteomic projects (von der Lieth, 2009).

4.2 Glycoproteomic experiment

Glycoproteomics is a branch of the glycomic research which includes all glycoconjugates with proteins and focuses mainly on the analysis of glycosylation occurring at Asn residues (*N*-glycosylation) or Ser/Thr (*O*-glycosylation). Glycoproteomic analysis of released glycans and glycopeptides provides two different types of information. These are the global identification on glycans and the localization of glycosylation sites in the amino acid sequences of proteins. Such a distribution of analysis has deep purpose because it reflects two different biological processes in the cell. The localization of glycosylation site is connected with the beginning of glycosylation process (often co-translational) while the final glycan structure is the result of maturation of a protein during the post-translational elongating and trimming mechanism in ER or GA (Karlsson & Packer, 2009).

Glycoproteomic experiment using as a cornerstone method MS is mainly carried out either with released glycans from glycoproteins or with glycopeptides. More traditional glycoproteomic approach employs SDS-PAGE for a separation of glycoproteins followed by enzymatic or chemical release of glycans and their chromatographic separation or MS analysis. Another option is to analyze glycopeptides obtained by in-gel digestion. This approach can be applied to a mixture of glycoproteins and proteins in the whole proteome as well as for enriched glycoproteins (Wilson *et al*., 2006).

Another way of glycomic analysis is based on the analysis of all released glycans from a protein mixture. This global analysis might be used as the first screening method, which can indicate the synthetic pathway of the glycosylation in the cell. By comparison of overall glycan profiles, it can be possible to find various abnormalities in glycosylation

which resulting from altered glycosylation machinery. These aberrancies may often cause various disorders and diseases. Relatively recently, glycomic strategies for indentification of glycopeptides were developed. The glycosylation site is determined by isolation of glycopeptides from the cell (Zhang *et al*., 2003; Kaji *et al*., 2003). Both of these approaches generate complementary data for research of the glycoproteome. During recent years, more sophisticated techniques, as labeling glycoconjugates by stable isotopes for quantification (Yuan *et al*., 2005; Xie *et al*., 2004) and 2D methods for measurement and quantification of MS data of glycans from LC-MS (liquid chromatography coupled with mass spectrometry) experiments (Niñonuevo *et al*., 2005), have appeared at the forefront.

4.3 Fragmentation of glycans

In the initial stage of using MS for analysis of glycosylation by methods FAB-MS/LSIMS (fast atom bombardment mass spectrometry/liquid secondary ion mass spectrometry) or traditional techniques of ionization by electron (EI; electron impact), it was observed that glycoconjugates such as free glycans and glycosfingolipides show significant fragmentation of the glycan structure together with the pseudomolecular ion. Based on these findings, a fragmentation nomenclature of glycans was created (Fig. 6, Domon & Costello, 1988) which, with minor updates (Karlsson *et al*., 1996), is still the basis for explaining of the processes taking place during MS of glycans. However, it is necessary to note that the nomenclature describes only fragmentation of glycans, but it does not predict fragmentation as such. The fragmentation pattern can be influenced by use of different fragmentation techniques, ionization modes (negative, positive), charge states of parent mass, type of mass spectrometer and by transfer of energy during a collision (Tab. 3; Morelle & Michalski, 2005).

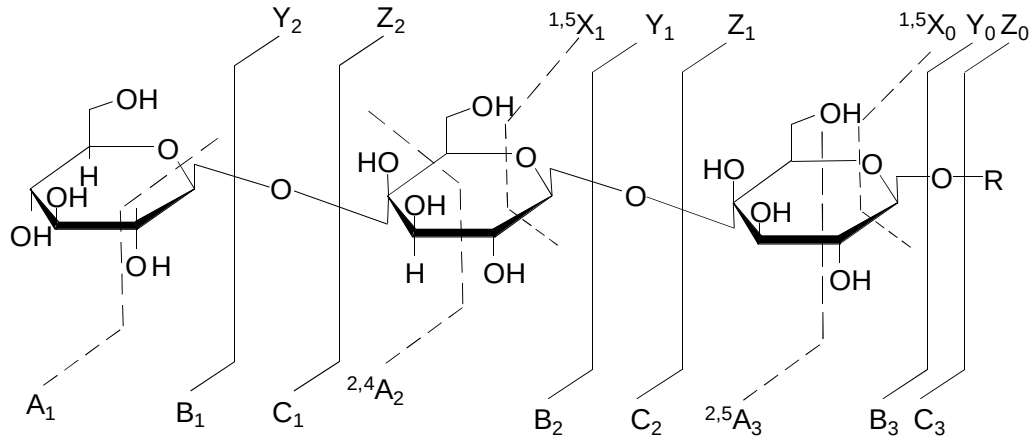


Fig. 6

Nomenclature by Domon and Costello describes the fragmentation of glycans. In this nomenclature, the ions having a charge on the non-reducing end are denoted A, B and C and the ions carrying a charge at the reducing end are X, Y and Z. A and X correspond to the

cleavage of the sugar ring while B, C, Y and Z correspond to the cleavage of glycosidic bonds. Subscripts indicate the position of cleavage starting from the reducing end for X, Y and Z ions and from non-reducing end for the other. In the case of the sugar ring cleavage, subscripts denote cleaved bond.
-reconstructed from Domon & Costelo, (1998) by means of software Chemsketch; (http://www.acdlabs.com/resources/freeware/chemsketch/)

Tab. 3
Parameters affecting the fragmentation of glycans (adopted from Morelle & Michalski, 2005).

Parameter	**Example**
Mode	Positive (+) and negative (-)
Charge state	$[M + H]^+$, $[M + 2H]^{2+}$, $[M + 3H]^{3+}$
Adducts	$[M + Na]^+$, $[M + 2Na]^{2+}$, $[M – 2H]^{2-}$, $[M + Cl]^-$
Derivatization	Permethylation, reduction, reductive amination of reducing termini
Energy transfer	CID, CAD (collision-induced/associated dissociation), ECD (electron capture dissiociation), PSD (post-source decay)
Collision energy	High energy collisions (>1 keV), low energy collisions (<0.5 keV)

Fragments of complex glycans give rise to two main types of ions which are the result of two types of cleavage: (a) the cleavage of glycosidic bonds which leads to rupture of the linkages between monosaccharide units, (b) the cleavage withing the circle breaking two linkages on one sugar residuum. Cleavage of glycosidic linkages gives information about monosaccharide sequence and branching. Cleavages of circles are usually less frequent and may be used for determination of linkages (Morelle & Michalski, 2005).

A significant effect on the fragmentation of the glycan arises from the type of parent ion. Decay rates of the protonated ions are faster than ions which are coordinated by alkali metals. Decay rates of the ions formed by alkali metals decrease in the order Li^+, Na^+, K^+ and Cs^+. Exothermicity of binding ion decreases in the same order suggesting that protonated and lithiated ions are formed with energy excess and these can be used for initiation of fragmentation. It was also observed that most branched glycans produce the lowest number of fragment ions. This phenomenon is attributed to coordination of the metal ion to sugar.

The smallest alkali metal produces the largest number of fragment ions. Presence of quasimolecular ion is thus dependent on the size of the metal ion and the size of the sugar. All these relationships are common features of fragmentation patterns for various ionization techniques (Ngoka *et al*., 1994; Cancilla *et al*., 1996).

4.4 MALDI MS fragmentation

MALDI MS instruments allow several different fragmentation techniques: in-source decay (ISD), post-source decay (PSD), and collision-induced dissociation (CID). ISD fragment ions originate from unstable molecular ions. These are rapidly disintegrated and correspond to ions produced within ion source before accelerating into a mass analyzer. Ions can be observed by TOF analyzer (Naven *et al*., 1997) or magnetic sector analyzer (Harvey *et al*., 1995). PSD ions correspond to fragments resulting from metastable ions which were extracted from the ion source. Fragmentation occurs between an ion source and a reflectron in the case of using TOF analyzer operating in reflectron mode. CID ions are formed from stable ions within a collision cell filled neutral gas. Parameters such as the laser wavelength and energy, extraction voltage and choice of matrix have a direct impact on the type and degree of ion fragmentation (Luo *et al*., 2002).

After using PSD technique on sodiated branched glycan molecules $[M + Na]^+$ released from glycoproteins, characteristic differences were observed corresponding to branching points and binding positions (Spengler *et al*., 1994). In PSD spectra, dominant fragments correspond to the cleavage of glycosidic bonds, whereas fragmentation of sugar ring is very rare. Therefore, the major fragment ions are B, C, Z and Y which provide information about the sequence and branching. In mass spectra from experiments using CID fragmentation, one can observe in addition to series B, C and Y many more fragments formed by breaking bonds within rings. The most important fragments are $^{1,5}X$ showing the overall constitution of the monosaccharide residues which provides valuable information about the sequence and branching. The presence of these fragments allows to identify Y ions and subsequently B and C, and thus to obtain information about the sequence of completely unknown non-derivatized neutral glycan (Harvey *et al*., 1997; Spina *et al*., 2000).

4.5 Mass spectrometry of released glycans

As mentioned earlier, electrospray ionization (ESI) and MALDI MS play a critical role in the characterization of glycosylation (Zaia, 2004). MALDI-TOF MS offers a relatively simple method for “screening” of complex mixtures. On the other hand, MS/MS methods using ESI and CID as a fragmentation technique are very suitable for the production of fragment ions that allow determining of the sequence as well as the linkages between glycans.

MALDI-TOF MS is very often used in the first step of an analysis. This is due to the unique ability of this method to quickly generate information about the nature and diversity of glycans released from native or recombinant glycoprotein or even more complex biological sample. Neutral glycans provide intensive signals in positive ion mode corresponding to the formation of molecular ions of type $[M + Na]^+$. This ion is often accompanied by a presence of weaker signal ion $[M + K]^+$. Analysis of sialylated glycans is more difficult in this way due to their formation of ion mixtures such as $[M + Na]^+$, $[M + K]^+$, $[M – nH + (n + 1)Na]^+$ a $[M – nH + (n + 1)K]^+$. Moreover, sialylated glycans can very easily lose a significant amount of sialic acids in ion source or after extraction of ions from ion source. This leads to the formation of poorly resolved fragments of metastable ions which are often observed in the MALDI-TOF spectra. In order to avoid these losses, sialylated glycans are analyzed in negative linear ion mode (Morelle & Michalski, 2007). Nowadays, several derivatization methods are used to improve a stability of sialic acides by neutralization of their negative charge. One of these methods can be methylesterification of carboxyl groups in sialic acids. Sodium salts of sialic acids react with methyl iodine which allows simultaneous analysis of neutral and sialylated glycans in positive ion mode (Powell & Harvey, 1996). Another method is permethylation of glycans using the techniques described by Ciucanu and Kerek which also stabilize sialic acids (Ciucanu & Kerek, 1984). There are several papers describing principles of this approach in considerably more efficient variants (Ciucanu & Costello, 2003; Kang *et al*., 2005).

Derivatization by permethylation remains the most important type of modification in MS of glycans and it offers several advantages: (a) very simple removal of salts using a lipophilic extraction; (b) significant improvements of sensitivity in detection of molecular ions; (c) allows simultaneous analysis of neutral and sialylated glycans in positive ion mode; (d) this approach leads to a predictable fragmentation which provides characteristic “map” of fragment ions for each sugar residue; (e) after MALDI-TOF MS analysis of permethylated glycans may be methylations also used for determination of all interglycosidic bonds by gass chromatography coupled with MS analysis (GC-MS) of chemically modified monosaccharides, which are obtained by methylation, hydrolysis, reduction and acetylation of glycans (Morelle & Michalski, 2007).

Analysis of linkages using methylation proceeds as follows. Firstly, all free hydroxyl groups of intact glycan are methylated. Then, the glycan chain is hydrolyzed which results in the formation of monosaccharides. Their subsequent reduction opens sugar rings and it forms sugar alcohols. Hydroxyl groups which took part in glycosidic linkages before hydrolysis are acetylated and the resulting mixture of volatile sugar derivates are analyzed by GC-MS. Fragmentation pattern for each sugar unit can be used for identification of positions of

various modifications. This information serves for identification of hydroxyl groups which were involved in the glycosidic bond (Maureen & Drickamer, 2003).

4.6 Analysis of glycopeptides

In order to solve the problem with suppression of MS signals of glycopeptides in the presence of non-glycosylated peptides, it was necessary to improve the methodology for enrichment of glycopeptides using different affinity methods. The use of specific proteases (usually trypsin) for preparation of peptide mixtures has advantages as well as disadvantages. One advantage is that if an analyzed protein is known as regards to its sequence, *m/z* of peptides can be easily predicted and the same sample is possible to use for protein identification and determination of glycosylation. The negative aspect of using trypsin is a limited control over the size of glycopeptides. Common tryptic peptides may have a size exceeding 2000 Da even much more (total mass of tryptic peptide and glycan; Karlsson & Packer, 2009). Separation and enrichment of tryptic glycopeptides by chromatography is very complicated due to different charge states and size of glycopeptide and glycan. Therefore, alternative methods were suggested for isolation of glycopeptides which use less specific proteases such as proteinase K or pronase. These enzymes cleave peptides near the glycosylation site, which in combination with the knowledge of peptide sequence and characterization of the released glycans presented in glycoprotein, allows to deduce the identity and site of glycosylation. The advantage of this approach is that smaller glycopeptides may be isolated by standard chromatographic methods used for analysis of glycan as the peptide backbone has a tiny effect on the chromatographic properties of the glycopeptide (An *et al*., 2003; Larsen *et al*., 2005).

Alternatively, for isolating of glycopeptides can be used hydrophilic interaction between glycans and solid phase, such as cellulose and sepharose as was demonstrated in the enrichment of large glycopeptides of fibronectin, which were then separated by reversed phase chromatography (Tajiri *et al*., 2005).

4.7 Analysis of intact glycopeptides

The main objective of the analysis of glycopeptides is to obtain simultaneously some information about the structural heterogeneity of glycans as well as the peptide sequence. In a glycoproteomic context, such analysis should be performed at the global level with several glycoproteins obtained simultaneously from one tissue. The first practical aspect of glycoprotein analysis is the fact that it is generally difficult to detect them by the use of standard techniques of peptide analysis. Major difficulties are connected with ion suppression of glycopeptides and elucidating of glycosylation heterogeneity on one glycosylation site. Even in the case, when peptide mixture is prepared from single

glycoprotein, glycopeptides are due to their heterogenous glycosylation typically seen in the mass spectrum with much lower intensities than non-glycosylated peptides (Karlsson & Packer, 2009). Using 2D gel electrophoresis, individual glycoforms can be resolved into several spots by their charge (sialic acid and sulphates) and size. They are usually recognizable as a sequence of spots with different values of pI and increasing acidity (Wilson *et al*., 2002). Such a pre-separation of various glycoforms results in more homogenous tryptic mixtures after a digestion.

High resolution MS belongs among very powerful tools for the analysis of glycosylation heterogeneity. With the aplication of the technique IRMPD (infrared multiphoton dissociation) it was demonstrated that FT (Fourier Transform)-MS can be used for sequencing of glycans from sialylated glycopeptides from cerebrospinal fluid (Håkansson *et al*., 2003a). LC MS in combination with different fragmentation techniques, such as IRMPD and/or electron capture dissiociation (ECD; or ETD – electron-transfer dissociation) together with a high resolution FT MS, are currently the most precise approaches for sequencing glycans and proteins from one sample (Håkansson *et al*., 2003b). ECD was also used for the analysis of *O*-glycosylated peptides containing multiple glycosylation sites. ECD preferentially cleaves the peptide backbone while IRMPD allows fragmentation of the glycan part of the glycopeptide (Renfrow *et al*., 2005).

In one study, the instrumentation of MALDI-QIT (quadrupol ion trap) was applied for analysis of enriched glycopeptide fractions. This technique used a sequential fragmentation in ion trap for sequencing of glycans (MS^2) and peptides (MS^3; Wada *et al*., 2004). There are several other papers describing the analysis of glycopeptides from purified proteins, which exhibit an enormous amount of information usually obtained by manual interpretation of MS data. However, practicing of such type of analysis in global scale is not only very time-consuming process, but also then data interpretation is difficult (Karlsson & Packer, 2009).

5. Objectives

The aims of this work are:

- application of a general workflow for analysis of *N*- and *O*- glycosylation of proteins from various biological sources
- optimization of a sample preparation for MALDI MS and MS/MS analysis of glycopeptides and glycans
- development of a microgradient device for purification and simple LC separation of peptide and glycan mixtures
- analysis of *N*-glycosylation in recombinant ZmCKO1 and plant CAOs enzymes
- analysis of clustered *O*-glycosylation in the HR of pIgA1 (Ale)

6. An overview of methods

Two plant *N*-glycosylated proteins (ZmCKO1, CAOs) and one human *O*-glycosylated protein (myeloma pIgA1-Ale) were analyzed (Papers I, II, III). Although the studied biological problem and motivation were different, the analytical workflow and overall strategy were similar. All methods and experiments are described separately in Papers I, II and III (Appendix). Generally, glycopeptides, enzymatically released *N*-glycans and deglycosylated *N*-glycopeptides were studied in order to analyze *N*-glycosylation in plant proteins. Combining data from these three information sources provided a comprehensive insight into the character of the studied *N*-glycosylation. MS/MS analysis of *N*-glycopeptides is a golden standard for colleting data related to particular glycosylation sites (*N*-glycopeptide contains information about the site of *N*-glycosylation and the structure of *N*-glycan). Nevertheless, some glycopeptides may be very low abundant or hardly detectable due to inefficient ionization, inefficient enzymatic digestion etc. For these reasons, the data obtained from other information sources were very valuable especially for the validation of glycopeptides and confirmation of glycan composition.

In the case of the study of *O*-glycosylation in the pIgA HR, only an analysis of tryptic *O*-glycopeptides was useful. Short clustered *O*-glycans in the HR, which vary only slightly in their structure (GalNAc or GalNAcGal), were presented in the single tryptic HR *O*-glycopeptide. Enzymatic or chemical deglycosylation of *O*-glycans would not provide new useful information. Fig. 7 summarizes all the analytical steps from Papers I, II and III into one generall analytical workflow.

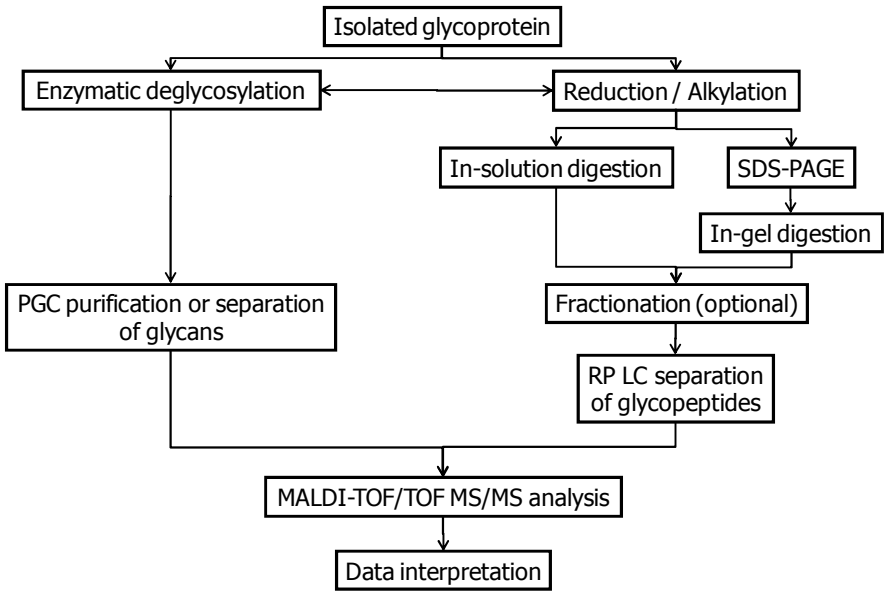


Fig. 7

The scheme displays a general analytical workflow for the analysis of protein glycosylation. Sample preparation is critical for MS analysis. An isolated glycoprotein was reduced and

alkylated (see Paper III about the role of Cys alkylation during the sample processing). Then, the sample was separated by SDS-PAGE or in-solution digested by trypsin (see Paper II where alternative proteases were also used). After SDS-PAGE, an in-gel digestion was perfomed. Prepared peptide mixtures (in-solution or in-gel digested) were pre-fractionated and/or separated by a microgradient device and directly deposited onto the MALDI target plate. MS analysis was carried out by MALDI-TOF/TOF MS/MS. Data were intepreted manually or, in the case of IgA *O*-glycosylation analysis, by means of the software BIOSPEAN. Complementary data were obtained after deglycosylation of isolated plant glycoproteins. Deglycosylated protein underwent the same procedure as native glycoprotein. Released *N*-glycans were separated from the deglycosylated protein by the microgradient device on PGC (porous graphitic carbon) column and analyzed on MALDI-TOF/TOF MS/MS instrument.

6.1 SDS-PAGE

SDS-PAGE allows separation of a protein sample from impurities and reduces complexity of peptide mixtures after the subsequent digestion by trypsin. It can also serve for monitoring (un)successful deglycosylation experiment and provides a preliminary overview of the sample heterogeneity (if mass differences between glycoforms are visible on the gel; see Paper I).

However, using SDS-PAGE may have also some disadvantages. One of them is related to an undesired modification of Cys residues in glycopeptides by residual acrylamide in the gel. This problem is discussed in detail in Paper III. Because SDS-PAGE is hardly reproducible, this method is not suitable for the purpose of absolute quantification. Moreover, the protocol comprising SDS-PAGE requires several manual steps and thus the sample is exposed to an increased risk of contamination with keratins, oxidation or other chemical modifications.

6.2 Fractionation and/or RPLC separation by use of a microgradient device – principles and development

A microgradient device was described for the first time in 2003 (Kahle *et al*., 2003). Authors constructed a simple chromatographic system allowing to separate samples using home-made short monolithic columns connected to a microsyringe. A non-linear gradient of ACN (acetonitrile)/0.1%TFA (trifluoracetic acid) was created by a specific aspirating of the mobile phase into a microsyringe (Fig. 8; Kahle *et al*., 2003). An elution of the sample was achieved by placing the syringe into a syringe infusion pump. Next, the same group used this system for purification and fractionation of peptide samples for MALDI-TOF/TOF MS (Moravcová, *et al*. 2009). A practical example of the microgradient creation and its stability

in time can be seen in Fig. 9. Six solutions with gradually decreasing ACN content were prepared. For visualization of the gradient in the syringe thionin acetate (TA) which was added into the ACN solutions was used. Time stability of the microgradient in the syringe was monitored for 2 hours (unpublished information by Dr. Pavel Řehulka).

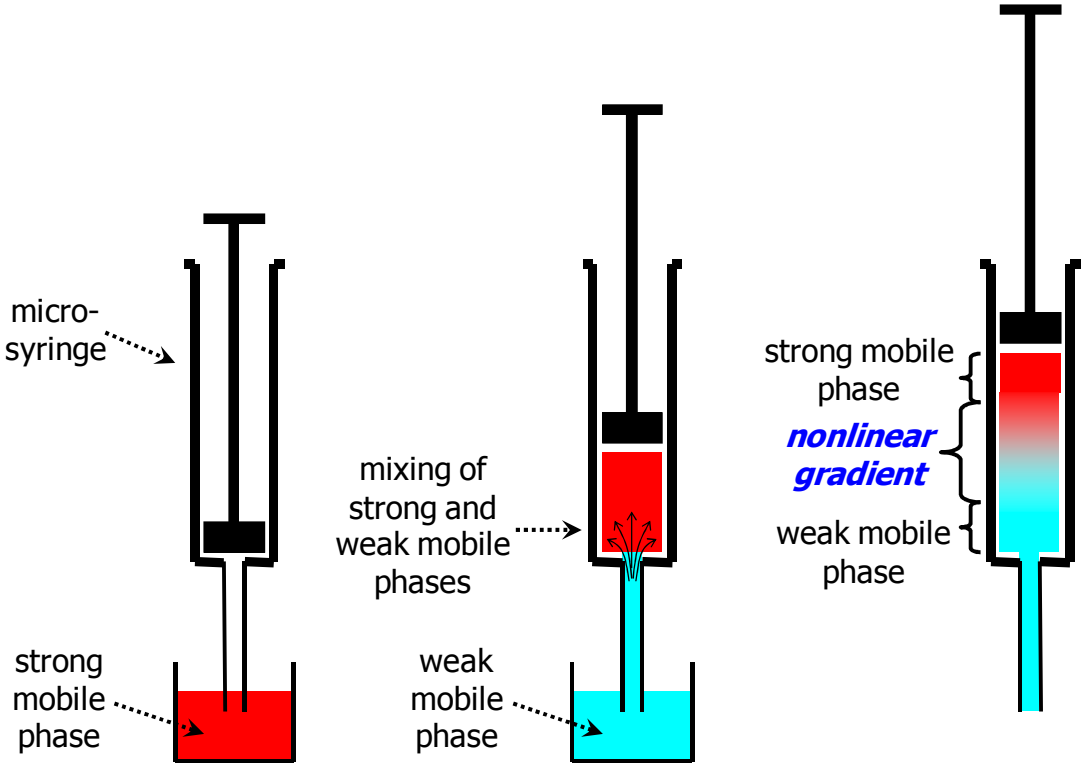


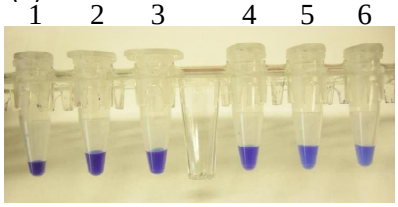
Fig. 8

The principle of the ACN gradient creation in a microsyringe. An S-shape gradient of ACN is formed inside the microsyringe as a result of turbulent mixing of the strong and weak mobile phases during the aspiration.

(a)

No.	ACN [%]	TA [ug/ml]
1	40	200
2	32	160
3	24	120
4	16	80
5	8	40
6	2	10

(b)



(c)

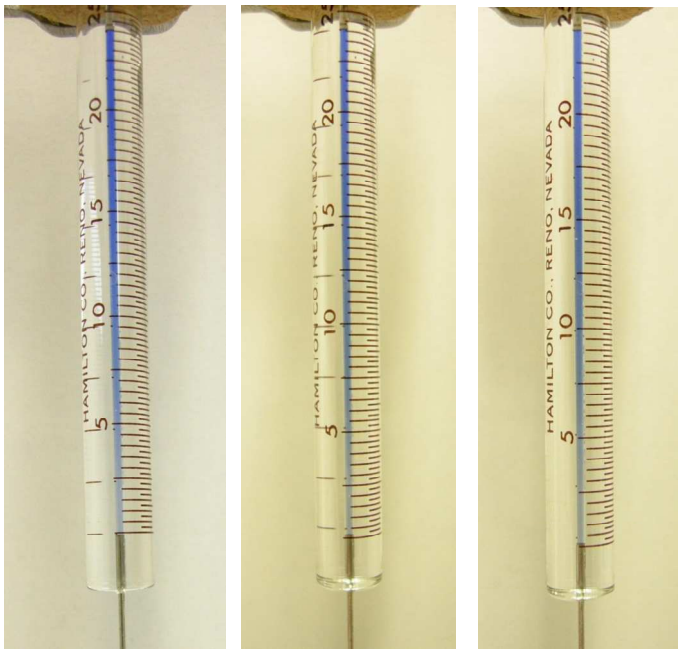


Fig. 9

Microgradient visualization in the microsyringe and its time stability. Table (a) shows the content of six mobile phases (b), which were consecutively aspirated into the syringe in the order from No. 1 to No. 6. The created gradient was monitored for 2 hours and during this time it remained stable (c).

A silimar system for fractionation and/or separation of glycopeptides and released *N*-glycans was used in Papers I, II and III. Although the original setup worked with short monolithic columns and the whole construction was slightly different, basic principles of the microgradient device were not changed. The apparatus most similar to the original system was used for the LC separation of ZmCKO1 *N*-glycopeptides and IgA1 HR O-glycopeptides (Paper I and III), where the only differences were the use of home-made capillary columns instead of monolithic ones and the use of a precolumn. Although the results obtained by the system using capillary columns were more than satisfactory, there were some disadvantages. Since the production of home-made capillary columns was time consuming and the whole system proved quite complicated, there was a necessity to simplify it. Capillary columns were replaced with fluorinated ethylene propylene (FEP) tubing columns which were filled with stationary phase (see Paper II). Significant advantages of these FEP columns are the convenient production and easier and faster replacement. Moreover, the whole system does not require the use of loading capillary and microtight unions for connecting loading capillary to a syringe and the analytical column to a loading capillary. Thus, the overall pressure in the system was significantly lowered which allowed separation on longer

columns and prolonged the lifetime of the syringe. Due to simple connecting microcolumns with each other via capillary pieces, it was also possible to easily purify and fractionate released glycans from glycoproteins or glycopeptides (Paper II). The use of an infusion syringe pump was another problem of the former system. The syringe infusion pump with an inserted syringe was set up for maintaining a constant flow rate. However, when the separation device was obstructed for any reason (overloading of the column, solid particles in the sample), the constant movement of the plunger destroyed the syringe due to an extreme increase of pressure in the system. Therefore, the syringe infusion pump was replaced with iron weights forming pressure which were held in a plexiglass tube. A modified version employs a lab stand which serves as a scaffold for holding all the main parts of the apparatus (a polycarbonate tube and a microsyringe coupled to a microcolumn; Fig. 10). A new construction of the microgradient device allows convenient and very simple fractionation of peptide mixtures and/or released glycans. It can also serve as preliminary fractionation of a sample prior to HPLC (high performance liquid chromatography) separation.

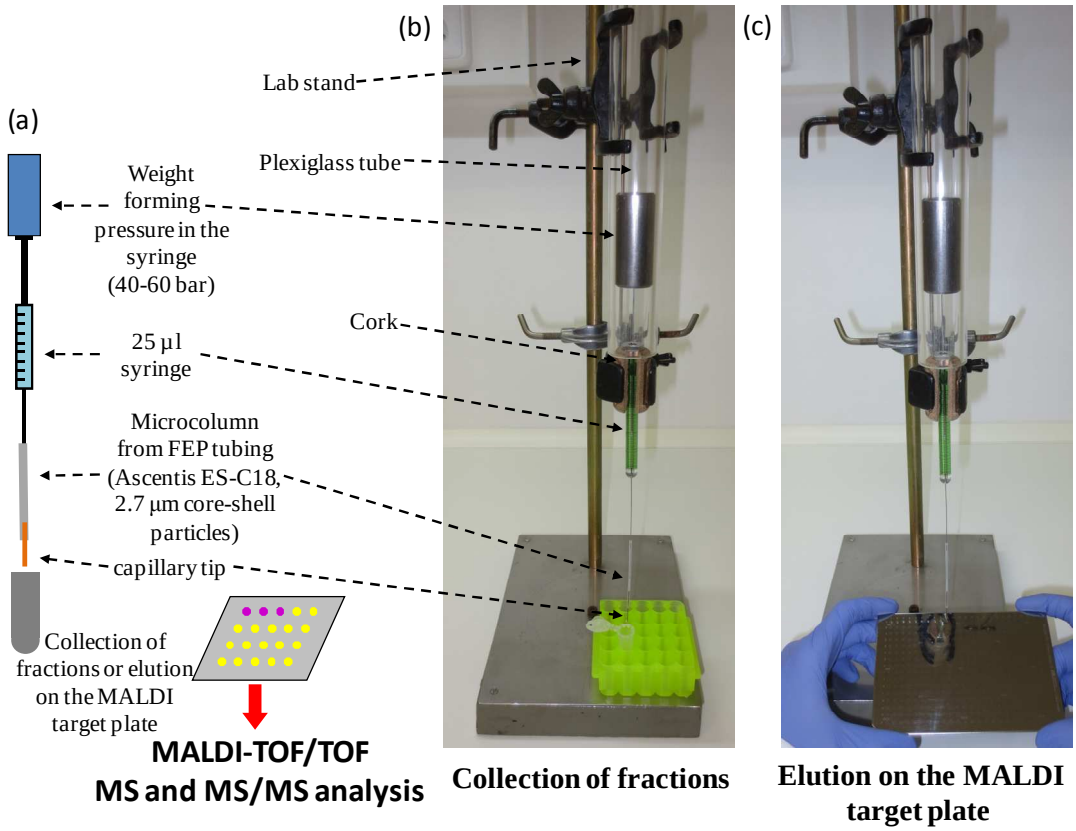


Fig. 10

(a) Major parts of modified version of the microgradient device. The developed system allows either convenient collection of glycopeptide fractions (b) or a direct elution on the MALDI target plate (c). Before the separation process, FEP microcolumn has to be wetted first with 80%ACN/0.1%TFA and then equilibrated with 2%ACN/0.1%TFA.

6.3 Details of preparation of analytical microcolumns

Preparation of analytical FEP microcolumns was described in Paper II; nevertheless, more comprehensive information about this process has not been published yet. Fig.11 dislays all parts necessary for the production of FEP microcolumns. Firstly, a 20-mm-long piece of silica capillary (360 μm OD and 20 μm ID) is cut by a ceramic capillary cutter. Secondly, it is inserted into a 100-mm long FEP tubing (1/16" x 0.25 mm ID) which was previously cut by a lancet and which has both ends gently widened with a steel pin. To the opposite end of this FEP tubing, a desired stationary phase is introduced in the form of a dense suspension in ACN. For this purpose, a white pipette tip (0.1-10 uL) connected to a plastic CombiTip (Eppendorf, Hamburg, Germany) is used. The suspension is firstly aspired to a white pipette tip connected to a CombiTip (approximately half the volume of the pipette tip). Next, the pipette tip is carefully introduced into the previously widened end of FEP tubing and the suspension is pressed down by pushing the CombiTip plunger forward. The sorbent particles are retained on the opposite site of the FEP tubing where the piece of silica capillary was previously introduced. When the FEP tubing is filled up with the stationary phase, the pipette tip is removed and 25 μL microsyringe filled up with 100%ACN is introduced into the widened end instead. By pressing down the mircosyringe plumber, the suspension is arranged into a dense homogenous column. Before use, new FEP tubing microcolumn has to be properly washed with 80%ANC/0.1%TFA.

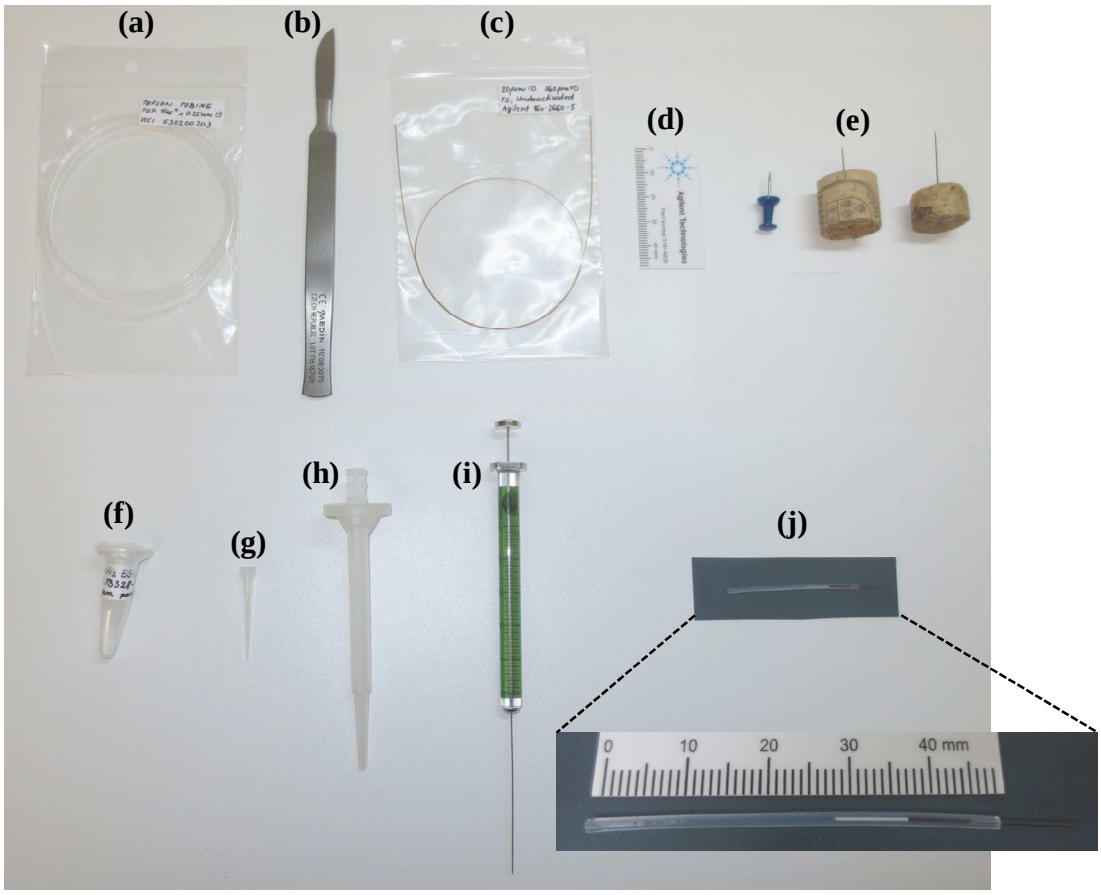


Fig. 11

Equipment for producing FEP tubing microcolumns. (a) FEP tubing (1/16” x 0.25 mm ID), (b) lancet, (c) silica capillary (360 μm OD and 20 μm ID), (d) ceramic capillary cutter, (e) steel pins, (f) suspension of stationary phase (Ascentis ES-C18 2.7 μm core shell in ACN), (g) white pipette tip (0.1-10 uL), (h) plastic CombiTip, (i) 25 μL gastight syringe, (j) ready FEP microcolumn.

7. Analysis of *N*-glycosylation in plant proteins

7.1 Paper I

Analysis of *N*-glycosylation in maize cytokinin oxidase/dehydrogenase 1 using a manual microgradient chromatographic separation coupled offline to MALDI-TOF/TOF mass spectrometry

Summary

The plant hormones cytokinins are substances which promote cell division and exert other important functions (Mok *et al*., 2000). Naturally occurring cytokinins consist of an adenine/adenosine moiety carrying an N^6-isoprenoid or N^6-aromatic side chain. The content of endogenous cytokinins depends on the balance between *de novo* synthesis, import and export rate, interconversion of distinct forms, transient inactivation by conjugation and catabolic reactions resulting in a complete loss of biological activity. Inactivation of cytokinins involves either *N*- or *O*-conjugation or irreversible oxidation by cytokinin oxidases/dehydrogenases (CKO/CKX, EC 1.5.99.12; Frébort *et al*., 2011). Due to a low concentration of CKO in plant tissues, purification of the native enzyme has often been demonstrated as an extremely difficult procedure requiring numerous chromatographic steps (Galuszka *et al*., 2001). The breakthrough came with the cloning of a gene coding for the isoenzyme 1 of maize CKO (ZmCKO1; Houba-Hérin *et al*., 1999; Morris *et al*., 1999), which consequently allowed identification of related genes in other species and their expression in heterologous hosts. A glycosylation in ZmCKO1 was initially deduced from its binding to lectin affinity columns and further confirmed by electrophoretic migration, which provided significantly higher molecular mass estimates than expected from sequence-based calculations (Houba-Hérin *et al*., 1999). The published crystal structures (Fig. 12) have shown that at least six of the predicted *N*-glycosylation sites may be occupied by glycans (Malito *et al*., 2004; Kopečný *et al*., 2010). However, the composition and size of the carbohydrate chains have not yet been investigated as well as their contribution to the activity and stability of the enzyme. MS has proven to be a reliable and invaluable tool for the analysis of *N*-glycosylation in plant proteins and elucidation of the corresponding *N*-glycan structures (Séveno *et al*., 2008). In this work, recombinant ZmCKO1 produced in *Y. lipolytica* was subjected to an enzymatic deglycosylation by endoglycosidase H under denaturing and non-denaturing conditions. The released *N*-glycans were analyzed by MALDI-TOF MS, ESI-Q-TOF (quadrupole-time-of-flight) MS and tandem MS (MS/MS). MALDI-TOF/TOF MS and MS/MS analyses were performed directly on *N*-glycopeptides generated by tryptic digestion of recombinant ZmCKO1. The obtained results revealed the

presence of high-mannose glycan structures and their importance for activity and stability of the enzyme.

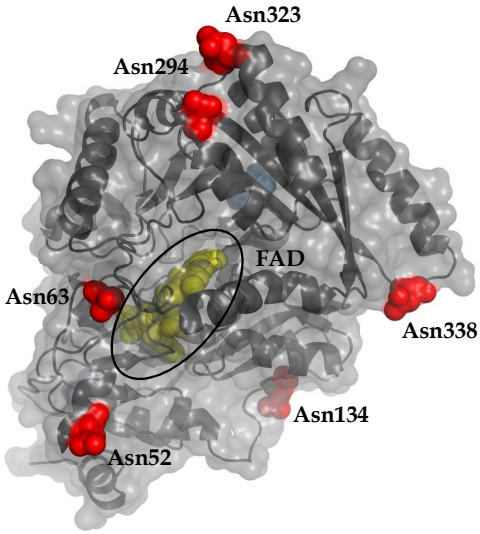


Fig. 12

Cartoon representation of ZmCKO1 molecule (PDB 3C0P). Six glycosylated Asn sites and FAD (flavin adenine dinucleotide) cofactor, both shown in spheres are colored in red and yellow, respectively. Black ellipse indicates the entrance to the active site.

7.1 Additional unpublished information or comments to Paper I:

N-glycosylation pattern of ZmCKO1 was analyzed. *N*-linked high-mannose glycans were released from the enzyme by endoglycosidase H treatment and their structure confirmed (see Paper I). The released *N*-glycans were subjected to a manual chromatographic purification followed by MALDI-TOF/TOF MS (Fig. 13)

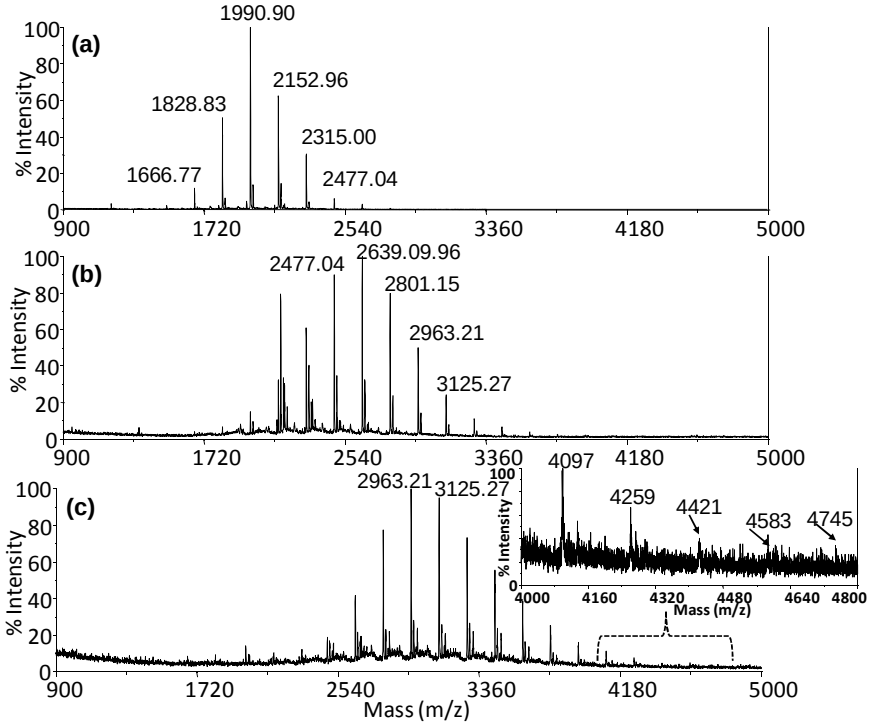


Fig. 13

MALDI-TOF/TOF mass spectra of neutral *N*-glycans released from ZmCKO1 by the treatment with endoglycosidase H. Pictures (a), (b) and (c) show three different fractions obtained by microgradient separation on PGC column of released neutral *N*-glycans from ZmCKO1. A glycan peaks series starting at *m/z* 1180 and goes up to *m/z* 4745 with a regular mass difference of 162 Da (sodium adduct peaks $[M + Na]^+$). The inset in (c) provides a magnification of the signals in the region of *m/z* 4000-4800. The specra were acquired on the ABI 4800 Proteomics Analyzer operating in the reflectron mode for positive ions; 3-AQ (3-aminoquinoline) was used as a matrix.

3-AQ (3-aminoquinoline) forms Schiff bases with the reducing end of sugars which results in derivatization accompanied by a mass increase of 126.1 Da (Rohmer *et al*., 2010). Taking this into consideration, the series of fractionated neutral *N*-glycans (Fig. 13) reflect the presence of glycan structures with an extent from $Man_5GlcNAc$ to $Man_{27}GlcNAc$ in the endoglycosidase H digest of ZmCKO1. These unpublished MS spectra support previous results in Paper I about presence of large *N*-glycans at Asn338 (more than 25 mannoses).

7.2 Paper II

Analysis of the glycosylation pattern of plant copper amine oxidases by MALDI-TOF/TOF mass spectrometry coupled to a manual chromatographic separation of glycans and glycopeptides

Summary

Plant diamine oxidases (EC 1.4.3.22) catalyze the oxidative deamination of diamines and polyamines which function as ubiquitous regulators in crucial physiological events such as cell differentiation and growth, wound healing, detoxification and signaling (Cona *et al*., 2006). From this point of view, it is obvious that pathways and reactions affected by diamine oxidase are rather multiple and insights to the structure of this enzyme could significantly improve understanding of them. The dimeric enzymes from pea seedlings (PSAO) and lentil seedlings (LSAO) have been investigated for a long time as typical members of the group. PSAO monomer has four potential *N*-glycosylation sites. Previous data from X-ray crystallography showed that two of them (Asn131, Asn558) were occupied and suggested that there might be a glycan chain bound at Asn334 in one of the two subunits of the enzyme. There was no evidence obtained for the presence of sugar residues at Asn364 (Kumar *et al*., 1996). An enzymatic deglycosylation of PSAO by endoglycosidase H under denaturing conditions combined with its proteolytic digestion by trypsin was carried out in order to analyze both *N*-glycans and "trimmed" *N*-glycopeptides with a residual *N*-acetylglucosamine attached at the originally occupied *N*-glycosylation site. The released *N*-glycans were subjected to a manual chromatographic purification followed by MALDI-TOF/TOF MS. For a direct determination of sugar binding and glycan structure determination, it was implemented a standard procedure which includes enzymatic digestion of purified native PSAO, fractionation of digests, RPLC chromatography using a manual microgradient device for glycopeptide separation and MS analysis of the separated glycopeptides. Glycopeptide sequencing by MALDI-TOF/TOF MS/MS clearly demonstrated *N*-glycosylation at Asn558. Manual spectra interpretation revealed the attachment of hybrid *N*-glycan chains and paucimannose structures. Such a glycosylation type has been reported for many plant proteins (Lerouge *et al*., 1998).

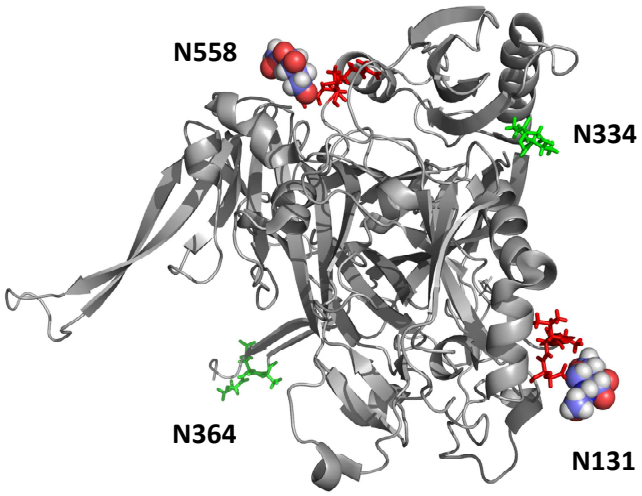


Fig. 14

The crystal structure of PSAO with highlighted glycosylation sites. There is only one subunit of the dimeric enzyme shown for clarity. The picture was drawn by use of PyMOL 1.2r1 (www.pymol.org) and coordinates from the PDB file 1W2Z (Duff, A.P. *et al*., 2004). The occupied glycosylation sites (131-NLS and 558-NRT) are shown in red and each of them bears two modeled *N*-acetylglucosamine units whereas the other glycosylation sites (unoccupied, 334-NGT and 363-NES) are shown in green.

7.3 Disscussion

Biochemistry and physiology of plant enzymes at Palacký University in Olomouc has a long term tradition. CKO1 and CAOs are typical subjects of scientific interest because both of these glycoproteins are involved in very complex methabolic pathways which are directly connected with fundamental principles of plant life at molecular level. For a deeper understanding of their function and position in particular biological process, it is necessary to know their molecular structure. For both enzymes, crystal structures were solved which provided clear evidence about the presence of glycosylation (Malito *et al*., 2004; Kopečný *et al*., 2010, Kumar *et al*., 1996). As mentioned previously, glycosylation imparts an additional level of information content to underlying polypeptide structures and alternates biological functions of glycoproteins. In mammals, *N*-glycans on glycoproteins are known to play crucial roles in many biological processes (Varki, 1993, Moremen *et al*., 2012). Far less is known about the biological role and biosynthesis of *N*-glycans in plants. Although the first stage of *N*-glycosylation mechanism has been strongly conserved during evolution, there are differences in the final trimming and decoration of *N*-glycoproteins in the GA between animals and plants (Rayon *et al*., 1998, Lerouge *et al*., 1998). For instance, high mannose type glycans in plants are identical to those found in mammalian cells but the formation of

complex type glycans differs in several aspects. Plant cells only synthetize bi-antennary *N*-glycans whereas the *N*-glycans in mammals can produce more branched structures. In plants, many complex type glycans lack the terminal *N*-acetylglucosamine residues which is probably result of different exoglycosidase activity during the transfer to the vacuole or following arrival to the vacuole (Lerouge *et al*., 1998, Fitchette *et al*., 1999). These differences and findings of new *N*-glycan structures led to formation of additional categories of *N*-glycans. As result, *N*-glycosylation of plant proteins is divided into four main categories according structure of attached *N*-glycan to Ans residue: 1) High mannose- type; 2) Complex-type; 3) Paucimannosidic-type and 4) Hybrid-type (Lerouge *et al*., 1998).

7.3.1 CKO1

The various CKXs family members seem to differ in their biochemical properties, namely in the regulation of their expression and in the subcellular localization of the gene products. Based on experimental findings which have been done on several CKX enzymes from various plant sources, protein glycosylation contributes to the regulation of enzymatic activity, translocation and/or protein stability. The glycosylation thus adds an additional level of complexity to CKX regulation (Schmülling *et al*., 2003).

In the Paper I, MS analysis of *N*-glycopeptides has showed recombinant ZmCKO1 enzyme as a heavily *N*-glycosylated glycoprotein. From nine potential *N*-glycosylation sites, six of them have been shown fully or, in the case of Asn134, partially occupied by high-mannose *N*-glycan chain. Kinetic experiments have clearly shown that although the loss of *N*-glycosylation after an enzymatic deglycosylation led to a destabilization of ZmCKO1 tertiary structure, the enzymatic activity was affected only slightly. The structural role of *N*-glycosylation was also confirmed by decreased thermostability of deglycosylated enzyme in comparison with the native glycosylated form. Because the studied glycoprotein was recombinant, it was not relevant to hypothetize the physiological function of *N*-glycosylation. Indeed, several studies have shown that glycosylation patterns in plant glycoproteins expressed in heterolougous systems often differed from the native enzyme forms (Rayon *et al*., 1996). Such findings underline the need to determine the structure of the glycosylated (control) protein against the new genetic background of the transgenic system.

An analysis of *N*-glycosylation of recombinant ZmCKO1 enzyme did not provide only information connected with the issue of CKXs. Current expression technology uses a variety of expression systems and the production of heterologous proteins is of considerable interest to basic research and biomedical and industrial applications. Many therapeutically interesting proteins are glycosylated which presents serious problem for their potential succesfull production by common expression organisms. *Saccharomyces cerevisiae* and other conventional yeast expression systems deal with certain limitations such as low

product yield, poor plasmid stability, difficulties in scaling-up production, low secretion capacities and hyperglycosylation (Madzak *et al*., 2004). Unfortunately, there are many differences in the glycosylation between yeasts and mammalian cells (Cereghino & Cregg, 2000). Generally, yeasts are not able to synthetize complex or hybrid glycan structures like mammals. Lower eucaryotes produce only mannose outer chains which further vary in length for endogenous and heterologous proteins and can be much longer than those in mammals (Goochee *et al*., 1991, Byrd *et al*., 1982). For example, *S. cerevisae* glycans are typically composed of 50-150 mannose units, a condition referred to as hyperglycosylation. Hyperglycosylated heterologous proteins may cause impaired activity and, as already mentioned, represent a significant problem for the pharmaceutical industry due to their possible exceeding antigenicity and rapid clearance. Therefore, alternative hosts for expression heterologous proteins are of considerable interest. *Yarrowia lipolytica* has been evaluated as a very promising host for expression heterologous proteins (Müller *et al*., 1998). Although the glycosylation pattern of proteins expressed in *Y. lipolytica* has not been extensively studied, human tissue plasminogen activator secreted from this yeast has been found to contain only short glycan chains (Buckholz & Gleeson, 1991). The presence of hyperglycosylation in ZmCKO1 enzyme, which was confirmed by MS, is the first experimental evidence of the post-translational formation of such long sugar chains in recombinant proteins produced in *Y. lipolytica*. *Y. lipolytica* has a high potential as an expression system due to its ability to produce more similar high-mannose type glycans to the mammalian. However, it should be noted that its glycosylation machinery is able to synthetize on some particular sites also hyperglycosylation (Paper I).

7.3.2 CAOs

PSAO and LSAO enzymes were subjects of interest in the Paper II. Both enzymes were isolated and purified from their plant sources according previously reported protocols (Lamplot *et al*., 2005; Floris *et al*., 1983). Because both enzymes were in their native forms and their glycosylation has not been investigated before, the analyzed *N*-glycosylation patterns provided information with potential to make new hypothesis about possible function of *N*-glycosylation of these enzymes and their biological roles which may involve binding of lectins in specific tissues during plant development and defense. Because all four potential *N*-glycosylation sites in PSAO structure are located on the surface of the molecule, deglycosylation or mutagenesis of the Asn residues would unlikely disrupt the structure (Kumar *et al*., 1996). MS analysis revealed hybrid and paucimannose type bound at Asn558. Moreover, presence of high-mannose type glycosylation in the PSAO structure was confirmed by analysis of neutral *N*-glycans released from PSAO by the treatment with endoglycosidase H. Such *N*-glycans in plant enzymes and other proteins has already been

described (Rayon *et al*., 1998), so it is not surprising that these were found also in PSAO and LSAO enzymes. The strinking resemblance to *N*-glycan chains observed in plant peroxidases exhorts to their comparison. Peroxidase as well as PSAO and LSAO are localized in the apoplast where they play important role in plant development and defence reactions. It has been suggested that hydrogen peroxid generated upon oxidation of polyamines can be utilized by peroxidase isoenzymes presented in the apoplast. CAOs act as hydrogen peroxide delivering system in the cell wall growth and differentiation. CAOs are ivolved also in defence mechanisms where hydrogen peroxide is key signaling molecule (Cona *et al*., 2006). It may be possible that both enzymes aminoxidase and peroxidase interact with the same proteins such as lectins during physiological processes (De Hoff *et al*., 2009).

8. Analysis of protein *O*-glycosylation

8.1 Paper III

Elucidating heterogeneity of IgA1 hinge-region *O*-glycosylation by use of MALDI-TOF/TOF mass spectrometry: role of cystein alkylation during sample processing

Summary

Many molecules involved in the innate and adaptive immune responses, including immunoglobulins, are glycoproteins. Altered glycosylation patterns of immunoglobulins occur in multiple autoimmune and chronic inflammatory or infectious diseases (Mestecky *et al*., 1993; Moore *et al*., 2005; Rademacher *et al*., 1994; Springer, 1997; Troelsen *et al*., 2007). Hence determining the glycan composition provides a better understanding of the disease mechanism. This work focuses on the *O*-glycosylation in IgA1 which becomes aberrant during IgAN. The HR of IgA1 binds *O*-glycans, which consist of GalNAc that may carry Gal and/or sialic acid. In IgAN patients, the heterogeneous IgA1 *O*-glycosylation has shown galactose deficiency, which is not yet fully understood (Mestecky *et al*., 1993; Novak *et al*., 2012). The sample preparation procedure used involved in-gel digestion of a pIgA1 (Ale) model and relied on a key step of optimized cysteine alkylation prior to SDS-PAGE, which allowed to avoid signal splitting of *O*-glycopeptides in MALDI-TOF/TOF MS. Separation of *O*-glycopeptides was achieved by RPLC using a microgradient device. MALDI-TOF/TOF MS of *O*-glycopeptides was completed by MS/MS. The acquired MS/MS spectra were interpreted manually and by means of a computational processing, which allowed assigning six *O*-glycosylation sites and revealed the existence of many isobaric *O*-glycoforms. The most abundant Gal-deficient *O*-glycoforms were $GalNAc_4Gal_3$ and $GalNAc_5Gal_4$ with one Gal-deficiency and $GalNAc_5Gal_3$ and $GalNAc_4Gal_2$ with two Gal-deficiencies. The most frequent Gal-deficient sites were localized at Ser230 and Thr236.

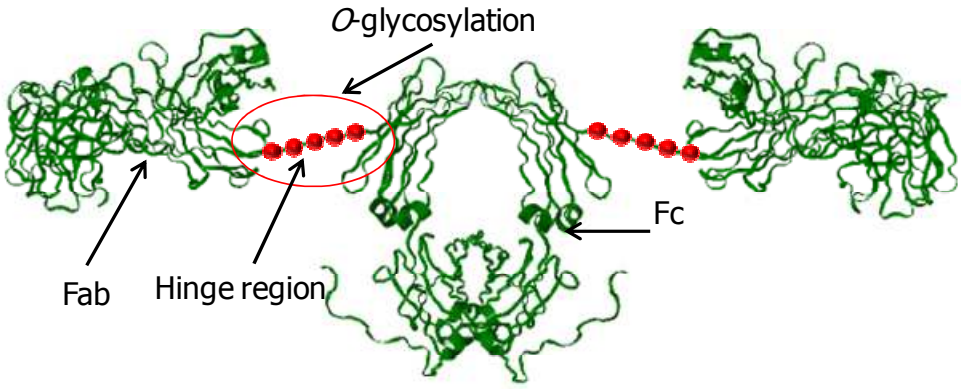


Fig. 15

The crystal structure of IgA1 monomer with highlighted *O*-glycosylation sites (red balls). The picture was drawn by use of Jmol Java wiewer (www.jmol.org) and coordinates from the PDB file 1IGA (Boehm. *et al*., 1999).

8.2 Additional unpublished information or comments to Paper III

Analysis of *O*-glycosylation of pIgA1 (Ale) has been perfomed. The acquired MS/MS spectra of *O*-glycopeptides were interpreted by combination of manual data inspection and by means of home-made software BIOSPEAN (Raus & Šebela., 2013; http://software.cr-hana.upol.cz/biospean/). BIOSPEAN is a freeware tool for processing spectra from MALDI intact cell/spore MS. The software interpretation of MS/MS was chosen due to very complex MS/MS data. Every single MS/MS spectrum of tryptic *O*-glycopeptide from IgA1 HR was mixture of several isoforms with the same *m/z* but with different sites of attachment and *O*-glycan composition at a given amino acid. Figures 16 and 17 provide additional information to Paper III for better understanding to the main principle of software interpretation.

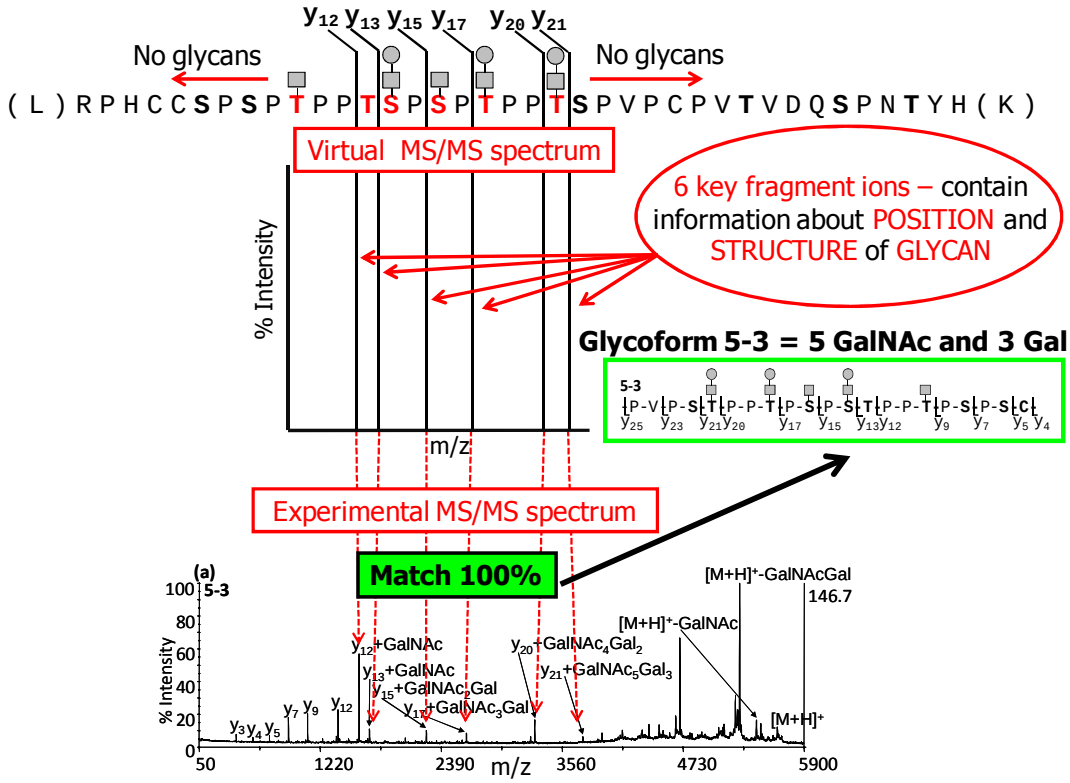


Fig. 16

Priciple of software inspection of MS/MS spectra of *O*-glycopeptides. More than 250 virtual MS/MS spectra were loaded into the BIOSPEAN. This database contained all possible isomers which could be theoretically constructed from six key fragment ions (y_{12}, y_{13}, y_{15}, y_{17}, y_{20} and y_{21}) from particular isoforms. As the scheme shows, software searches a "virtual MS/MS spectrum" from the database against an "experimental MS/MS spectrum". If the experimental MS/MS spectrum (in this case isoform 5-3; 5GalNac and 3Gal) contained the same combination of all six key fragment ions as it was in a virtual spectrum of isoform 5-3, the existence of this isoform was considered as highly probable.

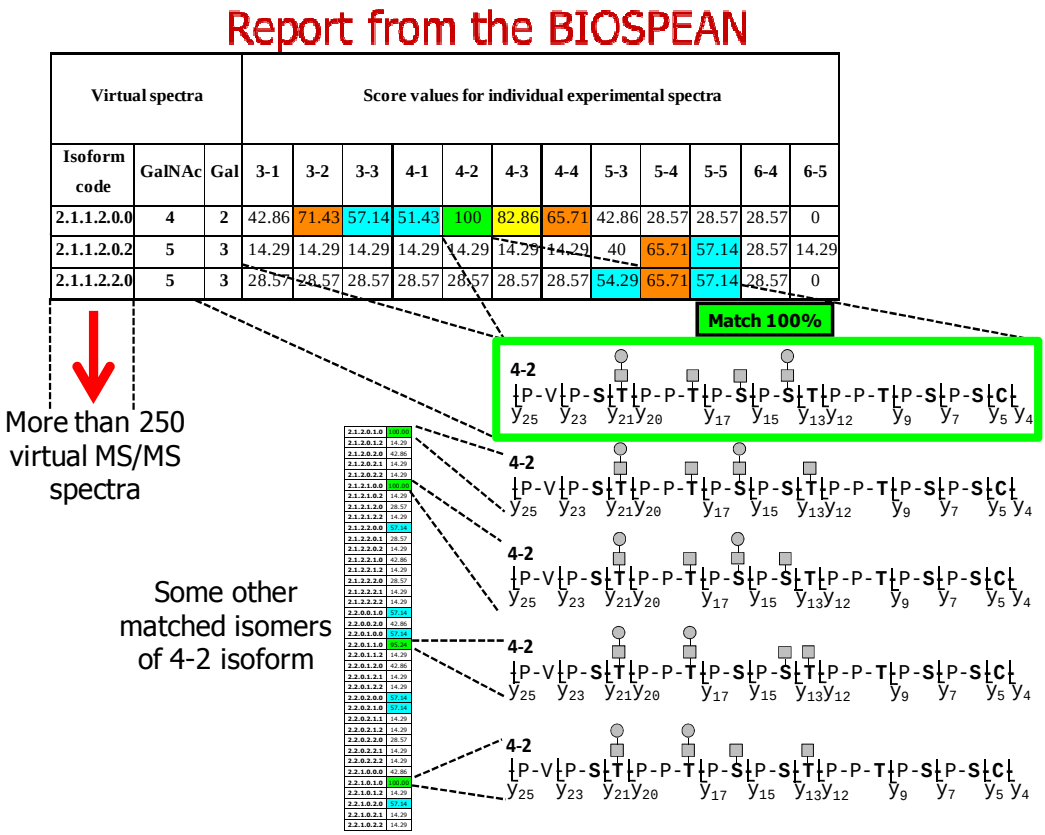


Fig. 17

For the software interpretation were selected 12 experimental MS/MS spectra of 12 isoforms (isoforms 3-1 up to 6-5). Fig. shows ability of the software to find all isoforms of particular *O*-glycopeptide (in this case 4-2). See Paper III for more comprehensive describtion of the table.

8.2 Disscussion

IgAN is probably the best understood disease where it is known that aberrant glycosylation plays key role in the pathogenesis. However, despite a considerable progress during the recent years, there are still many unclear processes on the molecular level, which need to be clarified in order to develop a non-invasive diagnosis, proper monitoring of disease progression and a disease-specific therapy (Wyatt & Julian, 2013). Aberrantly glycosylated IgA1 produced by patients suffering from IgAN, is a typical example of glycoprotein with clustered sites of *O*-glycosylation, i.e., Ser and Thr rich stretches within the amino acid sequence that have short *O*-glycan chains. These glycoproteins with clustered *O*-glycans are usually characterised by a distinc distribution of macro- and microheterogeniety (Renfrow *et al*., 2007). For example, IgA1 isolated from single source such as serum is thus always a population of variably *O*-glycosylated isoforms which differ from each other in terms of number of chains, sites of attachment and *O*-glycan composition at a given amino acid (Takahashi et *al*., 2012). Characterizing of these clustered sites and

understanding how the distributions change under different biological conditions or disease states are an analytical challenge. Heterogeneity of glycosylation leads to different physical, biochemical and biopharmaceutical properties and, therefore, also functional diversity. IgAN is characterized by prominent mesangial deposits of IgA1, co-deposits of C3 (Complement component 3) and IgG, IgM or both, typically associated with mesangial proliferation and expansion of the extracellular matrix. These mesangial deposits are derived from circulating immune complexes containting Gal-deficient IgA1. Although there is a great deal of evidence in the articles related to IgAN that this abberancy alone is not associated with a clinical expression of IgAN, Gal-defficient IgA1 protein species are directly involved in the pathogenesis of IgAN (Suzuki *et al*., 2011; Wyatt & Julian, 2013). MS analysis of aberrant IgA1 *O*-glycosylation pattern may lead to alternative methods for diagnosing and monitoring the disease as well as identifying targets for therapeutic intervention (Takahashi *et al*., 2012; Takahashi *et al*., 2010).

An important part of successful MS analysis (especially in MALDI techniques) is the sample preparation. MS analysis of *O*-glycosylation of pIgA1 HR (Ale) was hampered by undesired modification of reduced Cys residues in the HR tryptic peptide by a residual acrylamide during SDS-PAGE. Generally, these untargeted modifications; such as oxidation of Met (Chowdhury *et al*., 1995), formylation of –OH groups and/or *N*-terminus by formic acid (Aguilar, 2004), degradation of C-terminus, nonspecific alkylation by iodacetamid (IAM; *N*- and *S*-carbamidomethylation; Boja *et al*., 2001), deamidation of Asn and/or Gln (Wright, 1991), isomerization of Asp (Kameoka *et al*., 2003) or, as it is in this case, propionamidation of Cys; complicate MS analysis, because they bring additional complexity into the sample and lower detection limit of already low abundant (glyco)peptides. For these reasons, it is necessary to look at the established standard procotols with a certain amount of criticism and see various samples (glycopeptides) as individual analytical problems. The optimization of Cys alkylation of the pIgA1 HR tryptic peptide allowed successful MS/MS analysis and subsequent data interpretation.

PSD-MALDI TOF/TOF MS/MS analysis of pIgA1 (Ale) *O*-glycosylation revealed an unexpectedly high degree of microheterogeneity. Twenty *O*-glycopeptides were deduced from MALDI-TOF/TOF mass spectra of pIgA1 digest and the existence of another position isomers of *O*-glycoforms were suggested after computional inspection of MS/MS data by software BIOSPEAN. There is a supplementary table in the Paper III which shows more than 50 position isomers of *O*-glycopeptides from 3-1 up to 6-5.

An interesting discussion can be conducted on the topic of the initiation process of *O*-glycosylation sites and specificity of GalNAc transferases. Data proposed in Paper III suggest that the prevalent initial *O*-glycosylation site is at Thr228. This is in slight contrast with the data from Takahashi *et al*., 2010 where the dominant group of pIgA1 *O*-glycoforms

may originate from semi-ordered carbohydrate additions that proceed from Thr225 to Thr236. Nevertheless, very recent *in vitro* experiments have revealed that GalNAc-transferase more likely initiates *O*-glycosylation reaction at Thr228 (Stewart *et al*., unpublished data). Determinating initial *O*-glycosylation site is a very complex scientific problem which requires multi-displinary approach. A future explanation of *O*-glycosylation process could provide valuable information related not only to the IgAN pathogenesis but also general insight to the synthesis of mucin-like *O*-glycosylated proteins.

9. Conclusion

Analysis of PTMs of proteins has fundamental significance in general biochemistry and biology. This thesis is focused on one of the most common and important PTMs - glycosylation. During the recent years, major advances in sensitive high throughput technologies have been made in the fields of genomics and proteomics. Nevertheless, despite this technological progress, a study of protein glycosylation remains in many cases a great challenge.

This work presents a general strategy for analyzing glycosylation in various proteins isolated from different biological sources and provides a detailed insight into the MS and MS/MS interpretation of glycopeptides. Sample preparation is usually accepted as the most important step in mass analysis with MALDI mass spectrometers. A special attention was dedicated to a precise sample processing, especially Cys alkylation (Paper III) and simplification of peptide mixtures by fractionation and separation using a microgradient device. Separation of peptide mixtures provided more targeted glycopeptide analysis and partially helped to solve certain problems with ion suppression. The development and basic principles of the microgradient system were discussed and summarized in separate chapters (6.2 and 6.3). Data interpretation is an integral part of the MS analysis of glycopeptides. Due to a lack of suitable interpretation software, the evaluation of data was mainly based on manual inspection, although a very interesting and succesfull tool (Biospean) was used for interpretation of MS/MS spectra of IgA1 HR *O*-glycopeptides.

The biological impact of this work involves various fields. MS analysis of recombinant ZmCKO1 provided information about the structural function of *N*-glycosylation in this particular plant protein and extended knowledge about the glycosylation machinery of *Y. lipolytica* which is considered as a very promising alternative expression system. Structure analysis of *N*-glycans and *N*-glycopeptides in CAOs enzymes indicated their direct involvement in basic physiological processes in plants. The submitted thesis also presents results which could be applicable in biomedical research. Elucidating microheterogeneity in IgA1 HR showed a remarkable number of glycoforms and contributed to the deeper understanding of synthesis of clustered *O*-glycans and thus pathogenesis of IgAN.

List of abbreviations

3-AQ	3-aminoquinoline
ACN	acetonitilie
CAD	collision-associated dissociation
CAO	copper-containing amine oxidase
CFG	Consortium for Functional Glycomics
CID	collision-induced dissociation
CKO	cytokinin oxidase/dehydrogenase
ECD	electron capture dissiociation
EGF	epidermal grow factor
EI	electron impact
ER	endoplasmatic reticulum
ESI	electrospray ionization
ETD	electron transfer dissociation
FAB-MS/LSIMS	fast atom bombardment mass spectrometry; liquid secondary ion mass spectrometry
FAD	flavin adenine dinucleotide
FEP	fluorinated ethylene propylene
FT	Fourier transform
GA	Golgi apparatus
Gal	galactose
GC-MS	gass chromatography coupled with mass spectrometry
GPI	glycosylphosphatidylinositol
GT	glycosyltransferase
HPLC	high performance liquid chromatography
HR	hinge-region
IAM	iodacetamide
IgAN	IgA nephropathy
IRMPD	infrared multiphoton dissociation
ISD	in-source decay
IT/TOF	ion trap/time-of-flight
LC-MS	liquid chromatography coupled with mass spectrometry
LSAO	lentil seedling amine oxidase

MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
MS/MS	tandem mass spectrometry
OST	oligosaccharyltransferase
PCR	polymerase chain reaction
pIgA	polymeric myeloma immunoglobuline alpha 1
PSAO	pea seedling amine oxidase
PSD	post-source decay
PTM	post-translational modification
Q/IT	quadrupol ion trap
Q/TOF	quadrupole-time of flight
RPLC	revesed-phase liquid chromatography
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLD	soft laser desorption
TA	thionin acetate
TFA	trifluoracetic acid
TOF	time-of-flight
TSR	thrombospondin-1 type repeats
ZmCKO1	recombinant maize CKO1 isoenzyme

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Appendixes

Curriculum Vitae

Personal information	
Name	Vojtěch Franc
Sex	Male
Nationality	Czech
Date of birth	10th December 1986
Place of birth	Opočno
Address	Pitkova 593, Opočno 517 73
Phone	+420 737 058
E-mail	franc.v@centrum.cz
Skype	franc.vojta

Education	
2011 - Present	Ph. D. Centre of the Region Hana for Biotechnological and Agricultural Research, Palacky University Olomouc, Olomouc (Czech Republic)
2006 – 2011	M. Sc. Palacky University Olomouc, Olomouc (Czech Republic)
2002 – 2006	Grammar school diploma Gymnázium a SOČ Jaroměř, Jaroměř (Czech Republic)

Trainnings and research stays	
1st March – 26th August 2014 (6 months) (present)	Hungarian Academy of Science, Mass spectrometry group, Budapest (Hungary) research stay (supervisor: prof. Karoly Vekey)
16th June – 13rd December 2013 (6 months)	Department of Microbiology, University of Alabama at Birmingham, Birmingham (USA) research stay (supervisor: prof. Jan Novak)

1st March – 31st March 2011 (1 month)

19th November – 15th December 2012 (1 month)

18th February – 18th March 2013 (2 months)

Institute fur Chemische Technologie und Analytik
Technische Universitat Wienna, Wienna (Austria)
research stay (supervisor: prof. Gunter Allmaier)

31st July – 6th August 2011	5th European Summer School, "Proteomic Basic"; From methods to clinical applications in Brixen (Italy). Organized by Max-Planc-Institute for Biophysical Chemistry, Universitatsmedizin Gottingen; Ruhr-Universitat Bochum; Technische Universitat Munchen; Heinrich-Heine-Universitat Dusseldorf

Work experience

2012 – Present	Researcher Deparment of Protein Biochemistry and Proteomics, Centre of the Region Haná for Biotechnological and Agricultural Research, Palacky University Olomouc; Olomouc (Czech Republic)

Personal skills

Mother tongue(s) Czech

Other language(s)

UNDERSTANDING		SPEAKING		WRITING
Listening	Reading	Spoken interaction	Spoken production	
English B2	B2	B2	B2	B2
German A2	A2	A1	A1	A2

Levels: A1/A2: Basic user - B1/B2: Independent user - C1/C2: Proficient user
Common European Framework of Reference for Languages

Driving licence Type B

Participation on conferences

List of lectures:

Šebela M, Řehulka P, Kábrt J, Řehulková H, Oždian T, Raus M, **Franc V**, Chmelík J. Identification of N-glycosylation in prolyl endoprotease from Aspergillus niger and evaluation of the enzyme for its possible application in proteomics. Cukrblik 2009: Current Chemistry and Biochemistry of Carbohydrates, April 16, 2009. Masaryk University, Brno, Czech Republic. Lecture by M. Šebela. Abstract in: Abstract Book, Masaryk University, Brno, p. 18, ISBN 978-80-210-4831-7

Franc V, Rehulka P, Ozdian T, Sebela M. Studium proteinové glykosylace pomocí proteolytických a deglykosylačních enzymů a MALDI-TOF hmotnostní spektrometrie.Proteomics & Life, Sigma-Aldrich, Proteomická sekce CSBMB, Millipore a Beckman-Coulter , 30. března 2010. Lecture by M. Sebela

Sebela M, Koncitikova R, Lenobel R, **Franc V**, Rehulka P, Tylichova M, Kopecny D. Structural and functional study on the glycosylation in maize cytokinin oxidase/dehydrogenase 1, Proteomics & Life, Sigma-Aldrich, Proteomic section CSBMB, 22.3. 2011. Lecture by M. Sebela

Sebela M, Koncitikova R, Lenobel R, **Franc V**, Rehulka P, Tylichova M, Kopecny D. Structural and functional study on the glycosylation in maize cytokinin oxidase/dehydrogenase 1. IX Discussions in Structural Molecular Biology, Nové Hrady, 24. – 26. 3. 2011. Lecture by M. Sebela

Sebela M, **Franc V**, Rehulka P, Novak J. Analysis of protein glycosylation using a simple microgradient separation device combined off-line with MALDI-TOF/TOF MS analysis. 2. konference České společnosti pro hmotnostní spektrometrii, Fakulta vojenského zdravotnictví Univerzity obrany, Hradec Králové, October 17-19, 2012. Lecture by M. Šebela

Sebela M, Pecova M, **Franc V**, Dycka F. Hovězí trypsin není sám aneb další proteolytičtí gurmáni a jejich využití pro štěpení vzorku (Bovine trypsin is not alone or other proteolytic gourmands and their application for sample digestion). 2. Neformální proteomické setkání (2nd Informal Proteomic Meeting), 30th November – 1st December 2012, Olomouc, Přírodovědecká fakulta Univerzity Palackého. Lecture by M. Šebela

Řehulka P, **Franc V**, Šebela M, Končitíková R, Lenobel R, Kopečný D, Madzak C, Novák J. Analysis of Protein Glycosylation Using a Simple Microgradient Separation Device Combined Off-line with MALDI-TOF/TOF MS Analysis. 6th Central and Eastern European Proteomic Conference, Danubius Hotel Flamenco, October 14-17, 2012, Budapest, Hungary. Lecture by P. Řehulka

Franc V, Řehulka P, Raus M, Šebela M, Novak J. MALDI-TOF/TOF mass spectrometry study of O-glycopeptides from the hinge region of human IgA1: role of precise cysteine alkylation during sample processing 24th Mass Spectrometric Discussion-Meeting (MassSpec-Forum-Vienna-2013), 19th February - 20th February 2013, Universistat Wien. **Lecture by V. Franc**

Franc V, Řehulka P, Raus M, Stulík J, Novak J, Renfrow MB, Šebela M. Analysis of heterogeneous hinge-region O-glycosylation of human IgA1 using MALDI-TOF/TOF mass spectrometry. 11th Discussions in Structural Biology, Academic and University Center, Nové Hrady, March 14-16, 2013. Lecture by M. Šebela on 14th March. Abstract in: Materials Structure in Chemistry, Biology, Physics and Technology, Bulletin of the Czech and Slovak Crystallographic Association 2013, 20 (1):5

Franc V, Řehulka P, Raus M, Šebela M, Novak J. MALDI-TOF/TOF mass spectrometry study of O-glycopeptides from the hinge region of human IgA1: role of precise cysteine alkylation during sample processing 25th Mass Spectrometric Discussion-Meeting (MassSpec-Forum-Vienna-2014), 19th February - 20th February 2013, Universistat Wien. **Lecture by V. Franc**

List of posters:

Sebela M, Rehulka P, Kabrt J, Raus M, Rehulkova H, Ozdian T, **Franc V**, Chmelik J. Identification of N-glycosylation in prolyl endoprotease from Aspergillus niger and evaluation of the enzyme for its possible application in proteomics. 18th International Mass Spectrometry Conference, August 30 – September 4, 2009, Bremen, Germany. Poster PMM-241 by M. Šebela. Program, p. 71

Rehulka P, Lenobel R, Rehulkova H, Hernychova L, Sebela M, **Franc V**, Kahle V, Moravcova D. Separation of proteomic samples using simple microgradient device coupled

to ESI-MS. 28 th Informal meeting on Mass Spectrometry, 2.-6.5. 2010, Koszek, Hungary. Poster by P. Rehulka

Rehulka P, Kahle V, Moravcova D, Rehulkova H, Lenobel R, Sebela M, **Franc V**, Hubalek M, Hernychova L. RPLC separation of proteomic samples using simplemicrogradient device in combination with MALDI or ESI mass spectroscopy. 4th Central and Eastern European Proteomics Conference meets International Metabolomics Austria. Proteomics. Metabolomics, Vienna University of Technology, 29.8.-3.9. 2010, Vienna. Poster by P. Rehulka.

Franc V, Rehulka P, Sebela M, Novak J. MALDI-TOF/TOF mass spectrometry study of O-glycopeptides from the hinge region of human IgA1. "The 5th EU summer school in proteomics basic". 31. 7. – 6. 8.2011, Brixen/Bressanone, South Tyrol, Italy. **Poster by V. Franc**

Rehulka P, **Franc V**, Sebela M, Novak J. Analysis of O-glycosylation in the hinge region of human IgA1 using separation techniques and MALDI-TOF tandem mass spectrometry. "9th Austrian Proteomic Research Symposium". 8. – 9. 11.2011, Vienna, Austria. Poster by P. Řehulka

Koncitikova R, **Franc V**, Rehulka P, Sebela M, Lenobel R, Madzak C, Kopecny D. Mass spectrometric analysis of N-glycosylation in maize cytokinin oxidase/dehydrogenace 1. "5th Central and Eastern European Proteomic Conference". 19. – 22. 9. 2011, Prague, CZ. Poster by R. Končitíková

Koncitikova R, **Franc V**, Rehulka P, Sebela M, Lenobel R, Madzak C, Kopecny D.Analysis of N-glycosylation in maize cytokinin oxidase/dehydrogenace 1 using a manual microgradient chromatographic separation coupled offline to MALDI-TOF/TOF mass spectrometry. „2nd Meeting on „Metabolism, Signaling and Function of Cytokinin". 8. – 10. 7. 2012, Berlin, Germany. Poster by R. Končitíková

Franc V, Rehulka P, Sebela M, Novak J. Defining heterogeneity of clustered O-glycans from hinge region of human IgA by use MALDI-TOF/TOF mass spectrometry "Česká konference hmotnostní spektrometrie". 19. – 21. 10. 2011, Hradec Králové, CZ. **Poster by V. Franc**

Franc V, Rehulka P, Analysis of N-glycosylation in pea seedlings diamine oxidase using microgradient chromatography coupled offline to MALDI-TOF/TOF mass spectrometry. 6th Central and Eastern European Proteomic Conference, Danubius Hotel Flamenco, October 14-17, 2012, Budapest, Hungary. **Poster by V. Franc**.

Franc V, Rehulka P, Sebela M. Analysis of N-glycosylation in pea seedlings diamine oxidase using microgradient chromatography coupled offline to MALDI-TOF/TOF mass spectrometry. 23rd Joint Congress of the Czech and Slovak Societies for Biochemistry and Molecular Biology (23. biochemický sjezd), August 26-29, 2012, Masaryk University, Brno, Czech Republic. **Poster by V. Franc**.

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Franc V, Hargett A L, Hall S, Wei Q, Moldoveanu Z, Heath S L, Saag M S, Renfrow M B, Novak Protocol optimization for analysis of changes in N-glycosylation profiles of immunoglobulins from different sources by using separation techniques and mass spectrometry. 22nd Annual Microbiology Research Retreat October 18-20, 2013, University at Birmingham, The Alabama 4-Center, Columbiana, USA. **Poster by V. Franc**.

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