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BARLEY PROTEOMIC STUDIES RELATED TO BEER PRODUCTION

PROTEOMICKÉ STUDIE JEČMENE SOUVISEJÍCÍ S VÝROBOU PIVA

DIZERTAČNÍ PRÁCE
DOCTORAL THESIS

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Doctoral thesis assignment:

The main goal of the thesis is to investigate barley proteins and their changes that occur during the malting and brewing process. In addition, attention is paid to barley protein post-translational modification, namely enzymatic N-glycosylations and non enzymatic glycations.

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ABSTRACT

This work is focused on barley proteomic studies in relation to the beer production. Barley belongs between the most important crops in the world and its greatest use is for malting purposes, most commonly for the brewing industry. Studies of barley proteins during malting and brewing provide information about changes in protein composition or their post-translational modifications. Since the protein composition and their modifications are essential for the quality of malt and beer, barley proteomic studies have a potential to improve the malting and brewing process.

The main goal of this thesis is to investigate barley water-soluble proteins and their changes that occur during the malting and brewing process. The differences in protein composition were investigated using gel electrophoresis, reversed phase and size exclusion liquid chromatography, and MALDI-TOF mass spectrometry. The amount of some proteins is increasing and some new proteins are created in the germinated grain during the malting process. Contrary, many proteins are decomposed during the brewing process due to the high temperature and enzymatic activity of some proteases. Only some proteins belonging to the family of pathogenesis related proteins resist these harsh conditions and pass into the beer where they can influence several important quality properties.

Furthermore, various barley varieties and their differences were investigated. Varieties allowed for the production of certified Czech beer were compared to one variety with well-proven malting properties and one non-malting barley variety. In addition, alcohol-soluble barley proteins and their changes during the malting process were investigated as well.

A special attention was paid to selected post-translational modifications of proteins, namely glycosylations. Non-enzymatically glycosylated barley proteins (or glycated proteins) are formed during the malting process considering the large amount of glucose released from the starch degradation, and influence the protein stability as well as the beer quality, especially foaming properties. Enzymatic *N*-glycosylation represents the most frequently studied post-translational modification in plants because glycoproteins play a key role in various biological functions. Since glycoproteins are often present in a small amount, their enrichment from a complex mixture is required for their analysis. Lectin concanavalin A affinity chromatography was used for barley glycoproteins investigation. Moreover, the analysis of the carbohydrate part of glycoproteins was optimized.

This doctoral thesis brings important information about barley proteins, their modifications and analysis that are useful for further studies.

KEY WORDS

proteomics, barley, protein, brewing, malting, glycosylation

ABSTRAKT

Tato práce se zabývá proteomickými studiemi ječmene v souvislosti s výrobou piva. Ječmen patří mezi nejvýznamnější plodiny na světě a je využíván hlavně pro sladovnické účely, nejčastěji pro pivovarnictví. Studium proteinů ječmene během sladování a výroby piva poskytuje informace o změnách v proteinovém složení nebo jejich posttranslačních modifikacích. Jelikož jsou proteiny v ječmeni a jejich změny zásadní pro kvalitu sladu a piva, proteomické studie ječmene mají potenciál pro zlepšení procesu sladování a pivovarnictví.

Hlavním cílem této práce je studium ve vodě rozpustných proteinů ječmene a jejich změn, ke kterým dochází během sladování a výroby piva. Rozdíly v proteinovém složení byly sledovány pomocí gelové elektroforézy, kapalinové chromatografie na reverzní fázi, gelové chromatografie a MALDI-TOF hmotnostní spektrometrie. Během sladování se vlivem klíčení zrna zvyšuje množství některých proteinů a také jsou tvořeny nové proteiny. V průběhu vaření piva se naopak v důsledku vysoké teploty a enzymatické aktivity proteáz mnoho proteinů rozkládá. Těmto drsným podmínkám odolají jen některé proteiny, které přechází až do piva a mohou ovlivnit jeho kvalitu.

Dále byly zkoumány různé odrůdy ječmene a jejich rozdíly. Byly porovnány odrůdy povolené pro výrobu certifikovaného Českého piva s jednou osvědčenou sladovnickou odrůdou a jednou nesladovnickou odrůdou ječmene. Kromě toho byly studovány v alkoholu rozpustné proteiny ječmene a jejich změny v průběhu sladování.

Zvláštní pozornost byla věnována vybrané skupině posttranslačních modifikací proteinů: glykosylacím. Neenzymaticky glykosylované proteiny ječmene (neboli glykované proteiny) jsou tvořeny v průběhu sladování kvůli přítomnosti velkého množství glukózy uvolněné z rozkladu škrobu. Glykované proteiny ovlivňují stabilitu proteinů a kvalitu piva, obzvlášť pěnotvorný účinek. Enzymatické *N*-glykosylace představují nejčastěji studované posttranslační modifikace u rostlin, protože glykoproteiny hrají klíčovou roli v různých biologických funkcích. Glykoproteiny jsou často přítomny v malém množství, a proto je pro jejich analýzu potřebné obohacení glykoproteinů z komplexní směsi. Pro studium glykoproteinů byla využita afinitní chromatografie s lektinem concanavalin A. Kromě toho byla také optimalizována analýza sacharidové části glykoproteinů.

Tato disertační práce přináší důležité informace o proteinech ječmene, jejich změnách a analýze, které budou užitečné pro další studium.

KLÍČOVÁ SLOVA

proteomika, ječmen, protein, výroba piva, sladování, glykosylace

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DECLARATION

I declare that the diploma thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

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student's signature

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SUMMARY

1. INTRODUCTION

Proteins are abundant component in all cells and are important for various biological functions.[1] In the structural point of view, proteins are macromolecules consisting of one or more polypeptides, whereas each polypeptide consists of a chain of amino acids linked together by peptide bonds. The exact amino acid sequence is determined by the gene coding.[2] Proteins vary in molecular mass, ranging approximately from 5000 to more than a million Daltons (Da).[1] A scientific discipline dealing with the global analysis of proteins is called proteomics.[3] This term was established by Marc Wilkins and his colleagues in the early nineties.[4] Proteomics has grown rapidly in a short time and nowadays provides much information about living systems.[3]

Barley (*Hordeum vulgare* L.) is one of the most important cereal crops in the world. This highly adaptable cereal grain is produced from sub-arctic to subtropical climates. Historically, barley has been an important food source in many parts of the world. However, only 2 % of barley is used for human food at present, mainly in the developing world. It is used as an animal feed more likely, and the worldwide greatest use of barley is for malting purposes, most specifically for the brewing industry.[5,6] Malting is controlled germination of cereals evoking physical and biochemical changes within the grain, consequently stabilized by grain drying. Two types of barley are frequently used for malting: 6-row and 2-row. Two-row barley produces malt with a large extract, lighter colour, and less enzyme content than the 6-row type. Furthermore, hulled barley is preferred to hull-less barley for malting and brewing because the hull contributes to beer flavour and aids filtering during brewing.[6]

Whole barley grain contains about 10 – 17 % of protein. Protein is therefore a minor component in comparison to starch, which accounts for about 65 – 68 % of the grain mass.[6,7] Nevertheless, it is a major determinant of the quality of the grain for malting, brewing, and distilling. From a function point of view, barley proteins can be broadly classified into three groups: enzymes and enzyme inhibitors, storage proteins, and protective proteins.[7]

Although barley seed proteins have been investigated for a long time, the application of proteomic methods developed within the last two decades extremely enhanced the possibility to identify proteins of interest, to follow changes in protein composition during malting and brewing and also understanding the effect of protein modification on the quality of beer. Applications of proteomics in food science have therefore a great potential to improve significantly the malting and brewing process.[8]

Malting of barley has been carried out for centuries without knowledge of the molecular processes taking place in the seed during germination. Since the time maltsters have detailed insight into processes that occur during germination, malting is optimized for speed of germination and breeders are involved in the development of high-quality value barley varieties. The malt quality is predominantly determined by barley variety, namely its genetic background, and the physical conditions during growth in the field, harvest and storage of the grains. Thus, barley variety selected for use in the brewing industry must meet special quality requirements.[9,10]

In the Czech brewing, the traditional production of pale lager has been preserved due to historical reasons. The malting spring barley varieties suitable for the production of this Czech-type beer have a high level of residual extract, strong and full palate, excellent foaming power, and relatively low alcohol content.[11] The production of Czech-type beer with authentic mark "České pivo" (Czech beer) as a "Protected Geographical Indication" is allowed in the

Czech Republic by the European Union by Council Regulation EC No. 510/2006 of 20 March 2006[12]. The characteristic nature of the Czech beer is provided by a number of factors, including the raw materials used or the special brewing process. It is dominated by malt and hops, higher concentration of polyphenols and a higher pH value and no foreign tastes or odours are permissible. One very important characteristic of Czech beer is its bitterness that lingers in the mouth for a long time and also aids the digestive process.[12] For the Czech beer production, only few barley varieties are allowed (Tolar, Malz, Bojos, Blaník, Advent, Aksamit, Calgary and Radegast), according to recommendation of the Czech Research Institute of Brewing and Malting, PLC (RIBM).[13]

In this doctoral thesis, barley proteins were investigated using various proteomic techniques. The theoretical part contains general information about proteomics, post-translational modifications of proteins, beer production, important beer proteins, as well as the description of the analytical methods used for protein investigation. In the following chapters, the experimental and results of performed analyses are described and discussed. Protein composition was studied in barley grain, malt, beer and intermediate products of the beer production. Different barley varieties were also studied and compared, including those allowed for the Czech beer production, variety with well-proven malting properties, as well as one non-malting barley variety. A special attention was paid on post-translational modifications of barley proteins, specifically enzymatic glycosylations, and non-enzymatic glycations created during the malting and brewing process.

2. THEORETICAL PART

2.1. Proteomics

Proteomics is a rapidly growing area of molecular biology that is concerned with the systematic and large-scale analysis of proteins from cells, tissues or whole organisms. It is based on the concept of the proteome as a complete set of proteins produced by a given cell or organism under a defined conditions. Proteins present the major constituent of living cells and participate in almost every biological process in all organisms. Therefore, a comprehensive protein analysis provides a unique global perspective on how these molecules interact and cooperate to create and maintain a working biological system. Proteins are explored for use in many fields, including biotechnology, pharmacology, and biomedical applications.[3,14]

Table 1: Name, abbreviations, residue mass and side-chain composition of the twenty common amino acids[14,15]

amino acid	abbreviations		residue mass	side chain
	3 letters	single letter		
alanine	Ala	A	71.08	— CH_3
asparagine	Asn	N	114.10	— CH_2CONH_2
aspartic acid	Asp	D	115.09	— CH_2COOH
arginine	Arg	R	156.19	— $(CH_2)_3NH—C(NH)NH_2$
cysteine	Cys	C	103.15	— CH_2SH
glutamine	Gln	Q	128.13	— $CH_2CH_2CONH_2$
glutamic acid	Glu	E	129.16	— CH_2CH_2COOH
glycine	Gly	G	57.05	— H
histidine	His	H	137.14	— $CH_2—C_3H_3N_2$
isoleucine	Ile	I	113.16	— $CH(CH_3)CH_2CH_3$
leucine	Leu	L	113.16	— $CH_2CH(CH_3)_2$
lysine	Lys	K	128.17	— $(CH_2)_4NH_2$
methionine	Met	M	131.20	— $CH_2CH_2SCH_3$
phenylalanine	Phe	F	147.18	— $CH_2C_6H_5$
proline	Pro	P	97.12	— $CH_2CH_2CH_2$—
serine	Ser	S	87.08	— CH_2OH
threonine	Thr	T	101.11	— $CH(OH)CH_3$
tryptophan	Trp	W	186.21	— $CH_2C_8H_6N$
tyrosine	Tyr	Y	163.18	— $CH_2—C_6H_4OH$
valine	Val	V	99.13	— $CH(CH_3)_2$

Proteins are comprised of linear polymer chains of amino acids bonded together by peptide bonds. The sequence of amino acids is defined by the sequence of a gene. In general, the genetic code specifies 20 standard amino acids that are shown in the Table 1. Proteins are synthesized within the cell in a multi-step process which includes the transcription of DNA into RNA, the processing of RNA into mature mRNA, and finally translation of the mRNA into protein. The cell responds to internal and external changes by regulating the level and activity of its proteins. Moreover, many proteins are substrates for dynamic modifications that regulate their biological activity and interactions. Thus, the qualitative or quantitative changes

in the proteome and explanation of protein primary structure and post-translational modifications are crucial to studies of biological processes.

The proteome is a complex and dynamic entity. Therefore, proteomic studies calls for highly efficient and sensitive analytical methods for the identification, characterization, and quantification of proteins. Today, mass spectrometry (MS) is the most useful analytical technique for protein and proteome analysis because it provides a relatively simple platform for determination of the molecular mass as one of the fundamental properties of biological molecules. In this respect, several strategies can be taken depending on the complexity of the sample and the information required.[3,14]

2.2. Protein post-translational modifications (PTMs)

The central dogma of molecular biology affirms that a gene is transcribed into RNA and then translated into protein. It can be rephrased as the genome (all the genes in the organism) gives rise to the transcriptome (the complete set of mRNA in any given cell) which is then translated to produce the proteome (the complete collection of proteins in any given cell).

The transcriptome and proteome are much more complex than the genome because a single gene can produce many different mRNAs (e.g. by alternative splicing) and proteins. Different proteins can be generated by alternative use of start and stop codons and also, proteins synthesized from these mRNAs can be modified in various ways during or after translation. The same protein can be modified in many different ways giving rise to innumerable variants.[3]

Cell is not a static entity and it continuously responds to stimuli from its external and internal environments.[16] Therefore, almost all proteins are modified in some way during or after synthesis, either by cleavage of the polypeptide backbone or by chemical modification of specific amino acid side chains in the process known as post-translational modification (PTM).[3] PTM represents an important mechanism for diversifying and regulating the cellular proteome by providing more chemical properties than is possible using the 20 amino acids specified by the genetic code.[3,17]

Protein post-translational modifications play an important role in organism.[18] It greatly enhances functionality of proteins and regulates their activities, thereby largely increases protein complexity and dynamics. PTMs serve many different purposes in various cellular processes such as enzyme regulation, signal transduction, mediation of protein sub-cellular localization and stability, and interactions with proteins and other molecules.[3,19,20] Post-translational modifications are therefore essential for a cell's survival.[16] Importantly, inappropriate PTM is often associated with disease, and specific post-translational variants can be used as disease biomarkers.[3] Therefore, the analysis of PTMs, modificomics, is probably the most frequently studied area of interest in proteomic research.[18,20]

2.2.1. Types of protein PTMs

Post-translational modifications can be divided into two broad categories: first, covalent addition of one or more groups and second, hydrolytic cleavage of one or more peptide bonds in a protein by enzymes termed proteases (protein hydrolases).[21] More than 400 different types of PTMs have been documented.[3] Nevertheless, only a few of them have been extensively investigated at the proteome level.[17] The most commonly studied protein

post-translational modifications include glycosylation, phosphorylation, ubiquitylation, acylation, methylation, nitration, or acetylation.[18]

Some types of modification, such as glycosylation, are generally permanent. The most pervasive irreversible PTM is the proteolytic cleavage undergone by all proteins during their life cycles. Other PTMs, e.g. phosphorylation, are reversible and can be used to regulate protein activity in response to intracellular and extracellular signals and are thus often involved in signalling cascades. Post-translational modifications are therefore dynamic processes with a role in many biological processes.[3,21]

2.3. Glycosylation of proteins

Glycosylation is the covalent linkage of an oligosaccharide side chain to a protein.[22] In plants as in all eukaryotic cells, glycosylation represents the most frequently studied PTM.[23] The attachment of carbohydrates to a polypeptide backbone can strongly affect the physico-chemical properties of the protein, such as solubility, thermostability or protection from proteolysis. Glycoproteins play a key role in various biological functions and are important in many cell processes, including immuno-protection, virus replication, ligand-receptor interaction, cell growth, intracellular adhesion, occurrence of inflammation and so on. Aberrant glycosylation always results in the occurrence of diseases.[18,24]

A sugar moiety can be attached to a protein either during an enzymatic reaction, or a chemical reaction with no enzyme contribution.[24]

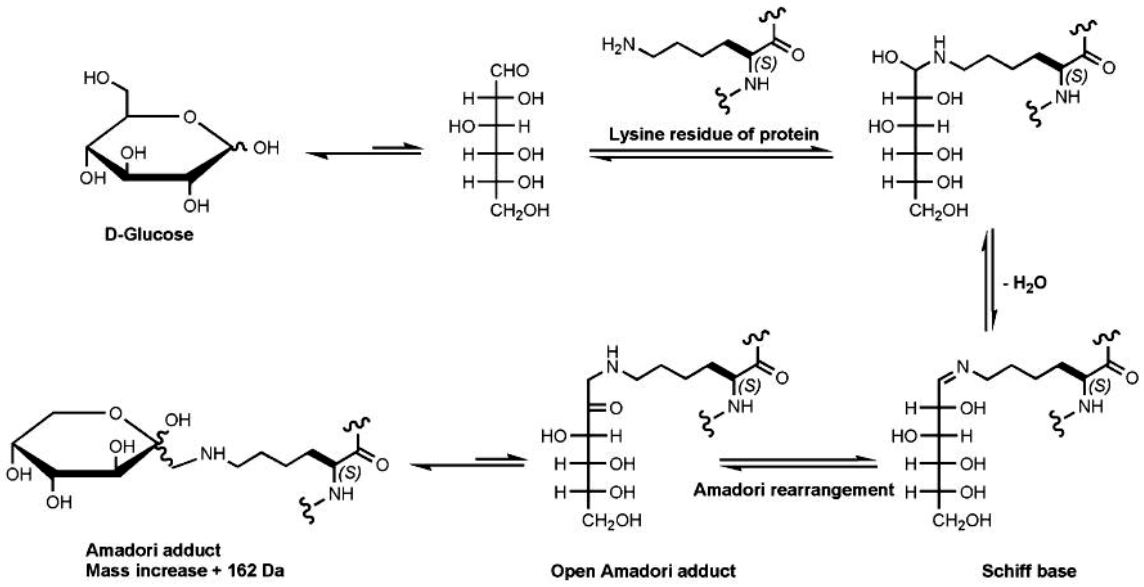


Figure 1: Scheme of non-enzymatic glycation[27]

Non-enzymatic glycosylation (glycation)

Glycation of proteins take place in the process known as Maillard reaction and leads to the formation of a heterogeneous group of compounds called advanced glycosylation end products (AGEs). This modification is involved in several age-related diseases in humans and in plants. AGEs are created by the reaction of reducing carbohydrates or their derivatives with free amine groups in peptides and proteins, such as amino groups in lysine, arginine or N-terminal amino acid residue.[22,25,26,27]

The proteins in barley malt are known to be glycated by D-glucose, which is a product of starch degradation during malting. The scheme of non-enzymatic glycation is shown in Figure 1. D-Glucose reacts with a free amine group yielding a Schiff base, which undergoes a rapid rearrangement forming more stable Amadori compounds.[27]

Enzymatic glycosylation

Various forms of enzymatic glycosylation are known depending on the linkage between the protein backbone and the oligosaccharide moiety:

- *N*-glycosylation – will be described in detail in the next chapter (2.3.1)
- *O*-glycosylation – *O*-linked oligosaccharide chain (glycan) is attached through an α-*O*-glycosidic linkage to the hydroxy-group of Ser or Thr nearing Pro. *O*-glycans often have lower mass than *N*-glycans structures, but can be more heterogeneous. *O*-linked glycosylation may happen at two cellular locations in the cell: in the Golgi, or in the nucleus and cytoplasm of cells. The nature of the *O*-linked glycans differs according to the location of the proteins. However, *O*-glycosylation of secreted proteins in plants is not as well understood as *N*-glycosylation.[18,23,28,29]
- *C*-mannosylation – *C*-mannosylation is the attachment of an α-mannopyranosyl residue to Trp via a C-C link and occurs on the first Trp in the motif W-X-X-W (or in some cases, W-X-X-C and W-X-X-F).[18]
- glycosylphosphatidylinositol (GPI) anchor attachments – GPI-anchored proteins are membrane bound proteins attached at their C-terminus to a trimannosyl glucosamine core structure. The reducing end of the latter moiety is bound to the hydrophobic region of the membrane via a phosphatidylinositol group.[29]

2.3.1. *N*-glycosylation

In case of plants, *N*-glycosylation is the most studied protein modification.[23] *N*-linked glycoproteins are expressed by all eukaryotic cells.[29] Oligosaccharides are attached through α-*N*-glycosidic bond to nitrogen of the amide group of Asn residues that are constituent of the potential *N*-glycosylation specific sequence N-X-S/T (where X is any amino acid except Pro). Although rare, the sequence motif A-X-C has also been shown to act as an acceptor site.[18,28]

N-glycans contain a common trimannosyl-chitobiose core $Man_3GlcNAc_2$ (mannose-N-acetyl-D-glucosamine, Figure 2a) with one or more antennae attached to each of the two outer mannose residues.[14]

There are three general types of *N*-glycans (Figure 2b-d):

- High-mannose *N*-glycans (or oligomannose) – contain only mannose (Man) residues (between five and nine) appended to the oligosaccharide core.
- Complex *N*-glycans – contain the additional monosaccharides, a variable number of lactosamine units and are often branched, resulting in a high-level of complexity. These first two are the most common group of the *N*-linked glycans.
- Hybrid *N*-glycans – possess structural features of both high-mannose and complex glycans.[14,29,29]

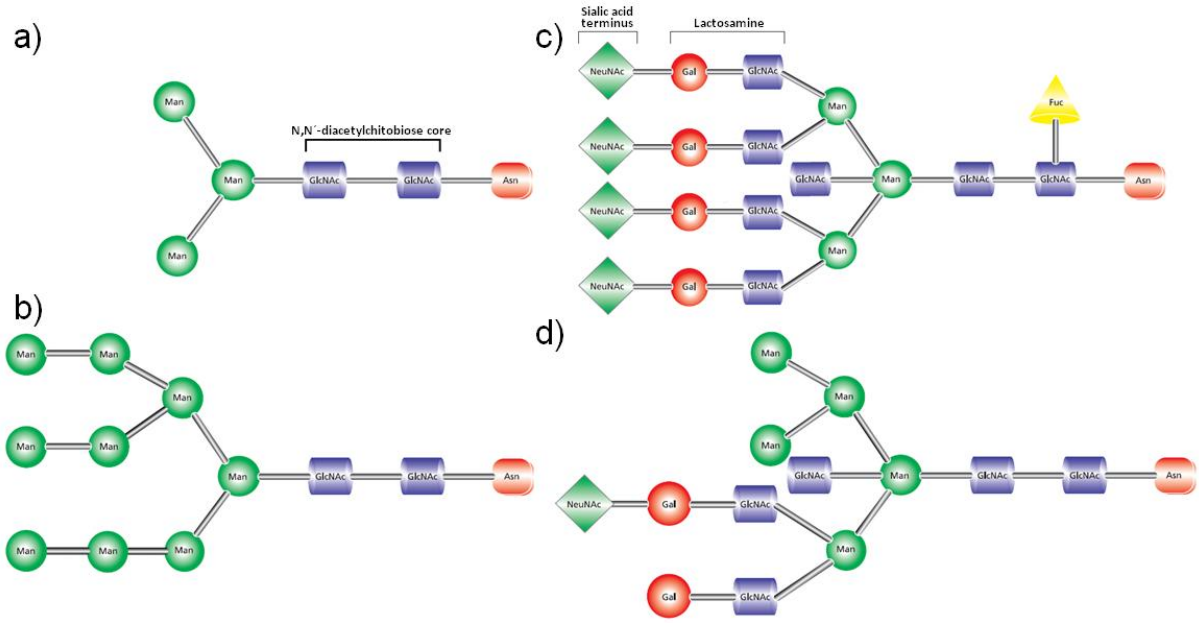


Figure 2: a) basic N-linked structure - pentasaccharide $Man_3GlcNac_2$; b) example of high-mannose N-glycan structure, c) example of complex (tetraantennary) N-glycan structure, d) example of hybrid N-glycan structure[29]

2.3.1.1. Formation of N-glycoproteins

N-glycosylated proteins are formed in endoplasmic reticulum (ER) as soon as the newly synthesized protein enters the ER lumen. First, an oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ (glucose-mannose-N-acetyl-D-glucosamine) is added onto Asn residue. Then, the glycoprotein moves from the ER through the Golgi apparatus to its final destination.[23] Within this pathway, the *N*-glycan undergoes several maturation steps involving the final removal and addition of sugar moieties by various glycosidases and glycosyltransferases.[22] More than 30 different types of sugar molecule can be added, and the structure and architecture of chains can vary significantly.[3]

The potential sequence N-X-S/T may occur many times along the polypeptide chain, but all the potential *N*-glycosylation sites does not need to be occupied. The glycosylation possibility depends on many factors, involving the tertiary structure of protein or occupancy of other glycosylation site. The same glycoprotein may be differently glycosylated under different conditions and the regulation of glycosylation pattern is not well understood.[28]

2.4. Beer production

Beer belongs to the most popular alcoholic beverages. For its production, four main raw materials are required: malt (mainly from barley grain), hop, water and yeast. Barley malt is a rich source of enzymes, like those responsible for the production of yeast substrates (amylases), flavour (lipoxygenases), or those involved in the formation of foam (will be further described in the chapter 2.4.2). Protein content is changing during the beer production and only about a third of the malt proteins pass into the final beer.[8,30]

2.4.1. Malting and brewing process

Malting is the first and essential phase of the beer production defined as the controlled germination of grain. Grain is thereby converted into the enzyme rich malt and the main aim of the malting process is the production and activation of enzymes.

Malt production process includes three steps: steeping, germination and malt kilning. Water content is increasing in grain during the steeping step (from 12 % to at least 40 % of moisture), what initiates enzymatic reactions and grain germinating. Germination is the main stage of malting, when embryo is growing and new enzymes are activated and formed. Amylolytic enzymes (α-amylase and β-amylase) are the most important enzymes formed during malting because they are involved in hydrolysis of starch, glycogen and other polysaccharides containing α1-4 glycosidic bonds. While the presence of α-amylase in barley grain has not been proven and this enzyme is formed during malting, β-amylase is already present in the barley grain and its content increases during malting. These starch degrading enzymes play a crucial role mainly during the mashing because they are responsible for the increasing amount of fermentable sugars in sweet wort. Other enzymes also contribute to the hydrolysis of β-glucans and hordeins (alcohol-soluble proteins), which would otherwise restrict access of enzymes to the starch granules. Germination typically takes about 5 days to obtain green malt. During the final kilning phase, germination is stopped by hot air, thus converted into the more stable and keepable form. This process is followed by the germ removing, malt refining and milling. The results of the malting process include an increase in enzyme activity and soluble protein, along with breakdown of starch into simple sugars and development of the typical colour and flavour. Moreover, some proteins are digested by active proteolytic enzymes during the malting and mainly mashing.[6,9,31]

The next step of the brewing technology is mashing. During the mashing process, polysaccharides present in the malt are enzymatically degraded and all desirable compounds of malt extract are converted into solution due to the increasing temperature.[6,31] The mashing program is running in several steps and is set up to hold the temperatures according to the optimal conditions of individual enzymes.[32] The obtained sweet wort extract is then separated from insoluble residues of malt. In the next step, sweet wort is boiled with hop, what results in dissolution of hop bitter and flavour substances and in product stabilization. During the hop boiling, the pH value is decreasing, which influence importantly the protein coagulation.[31] Furthermore, all of the microorganisms that might be found in wort are killed during boiling.[33]

Acquired wort is then cooled down and separated from sediment, thus prepared for the fermentation process. Wort fermentation is promoted by brewer's yeast *Saccharomyces cerevisiae* that cause the controlled conversion of saccharides into alcohol and CO_2 as well as production of required organoleptic properties of beer.[31] Fermentation also tends to produce flavours that are considered undesirable in finish beer. For this reason, beer must undergo some form of maturation, also referred as conditioning, lagering or aging. Immature beer is often referred to as "green beer" because it sometimes has the aroma of green apple, the result of elevated levels of acetaldehyde. During maturation, all of these undesirable compounds are reduces by the continuing action of the yeast. Furthermore, beer becomes carbonated because the yeast continues to give off CO_2.[34] In the end of the brewing process, beer is separated from yeast by sedimentation and filtered for removing of haze-forming particles and remaining yeast cells. Thereby, the product is stabilized and can be bottled and stored.[31]

2.4.2. Quality of beer

In brewing technology, the formation, stability, and texture of foam on the surface of beer are important aspects of beer quality and are critical for the consumer's visual estimation of beer.[8,35] The foam averts the volatilization of flavour and inhibits oxidation by prevention of the direct contact between air and beer. Beer foam quality is defined by its stability, creaminess, whiteness, intensity and lacing.[36] Moreover, excellent foaming power is also an important characteristic of Czech-type beer.[11]

Various components determine beer foam quality, such as proteins, lipids, polysaccharides, melanoidins, iso-R-acids, metal ions, alcohol as well as gas composition. The foam formation and stability favourably influencing components include proteins from malting barley and bitter substances from hops, while lipids and yeast proteins (e.g. thioredoxin[37], proteinase A[36]) are foam destabilizing components. Between important proteins with regard to beer foaming belong mainly protein Z, non-specific lipid transfer proteins (ns-LTPs) and protease/α-amylase inhibitors.[8,27,35]

Further characteristics influencing the quality of beer include beer colloidal haze and gushing. Colloidal haze can considerably reduce beer quality and also negatively influence the consumer's visual opinion on a beer because it is considered to be a sign of aging or contamination.[8] A variety of causes of haze formation are known, but the most frequent is protein-polyphenol interaction.[38] Gushing, defined as the over-foaming of beer upon the opening of the bottle, is a very serious quality defect. Gushing can be related to the quality of malt or probably to surface-active molecules.[8] The primary beer gushing is associated with the infestation of grain with microscopic fibrous fungi.[39]

2.5. Important barley proteins

Proteins and peptides contained in beer originate mainly from barley seeds; however, minor proteins from *Saccharomyces* species are also present.[40] The protein content in barley grains represents approximately 10 – 17 % of its total mass and about a third of the barley proteins pass into the final beer.[6,8] Proteins are essential for the quality of malt and beer.[9] As mentioned before, high-protein content decomposes available carbohydrates into fermentable sugars, proteolysis is necessary for yeast metabolism, and finally, proteins are important in beer foam retention and stability.[6]

2.5.1. Pathogenesis-related proteins (PRs)

The mature barley seed proteome is rich in pathogenesis related proteins (PRs). This large group of seed proteins is assumed to be involved in plant defence.[8] PRs are classified into 17 families, based on primary sequence, immunological relationship and biological activity, and numbered in the order in which they were described (Table 2). PRs are present in many plant species and can be found in all plant organs.[5] While most of barley seed proteins are precipitated upon unfolding or degraded by proteases during the mashing and wort boiling process, certain PRs resist these harsh conditions due to resistance towards proteolysis and thermal stability. Therefore, the majority of the beer proteins have been identified as PRs.[8]

Table 2: Families of pathogenesis-related proteins (PRs)[5]

Family	Properties
PR-1	antifungal
PR-2	beta-1,3-glucanase
PR-3	chitinase I - II, IV - VII
PR-4	chitinase type I, II
PR-5	thaumatin-like protein (TLP)
PR-6	protease inhibitor
PR-7	endoproteinase
PR-8	chitinase type III
PR-9	peroxidase
PR-10	'ribonuclease-like'
PR-11	chitinase, type V
PR-12	defensin
PR-13	thionin
PR-14	lipid-transfer protein (ns-LTP)
PR-15	oxalate oxidase
PR-16	'oxalate oxidase-like'
PR-17	unknown

Plants do not contain adaptive immune systems against pathogenic microorganisms and pests, but they have a lot of innate defence mechanisms for protecting dormant and germinating seeds, including the action of PRs. Barley natural resistance mechanisms involving PRs are important for barley producers as well as for the malting and brewing industry because infection of barley is extremely undesirable due to the adverse effect on the beer quality and the usage of pesticides and insecticides is inadvisable.[5]

PRs influence a beer consumer's health and beer quality in various ways:

- play a major role in the formation, stabilization and retention of foam
- may influence brewer's yeast fermentation
- have an impact on beer colloidal haze
- may influence the degradation proteins and carbohydrates during malting and mashing
- increase the beer nutritional value as a rich source of essential amino acids
- may have antioxidant activity
- may improve lipid metabolism
- may cause allergic reaction[8]

2.5.1.1. Protease/α-amylase inhibitors

Protease inhibitors (PIs) or PR-6 constitute the largest group of proteins identified in barley, malt and beer.[5,8] These proteins probably play a role in controlling the activity of barley proteinases during germination and they possibly protect the seed and young plant from microbes or pests by enzymes inhibition.[41] Various important proteins belong between barley seed PIs: bifunctional α-amylase/subtilisin inhibitor (BASI), chymotrypsin/subtilisin inhibitors (CI-1 A, B, C and CI-2A), trypsin/α-amylase inhibitors CMa-e, barley dimeric α-amylase/trypsin inhibitor (BDAI), serine protease inhibitors (serpins or protein Z4, Z7), etc.[5,8] A decrease of inhibitor amounts has been observed during germination by Maeda et al.[42] Protease/α-amylase inhibitors are not extensively glycated during the malting and brewing process, nevertheless, BDAI represent an exception. This inhibitor probably

undergoes structural modifications including glycation and phosphorylation.[5,8] Moreover, BDAI is present in the beer foam and contributes importantly to the beer foam stability.[36,37]

Glycosylated members of the α-amylase inhibitor family (BMAI-1, BTAI-CMb) are known to act as sensitizing agents in humans upon repeated exposure and were found to be the strongest allergens associated to beaker's asthma disease.[43,44] They are particularly reactive both in vivo and in vitro. These major glycoprotein allergens carry a single asparagine-linked complex glycan that contains both β(1-2) xylose and α(1-3) fucose. The xylosyl residue and, to a lesser extent, the fucosyl residue are key IgE-binding epitopes and largely responsible for the allergenicity of these and unrelated proteins from plants.[45,46]

2.5.1.2. Serpins (serine protease inhibitors) - Protein Z

Serine protease inhibitors can be classified in the group of PR-6 proteins (protease inhibitors).[5] Serpins were not originally classified as PRs; however, for their probable defensive role hypothesized on the basis of irreversible inhibition of exogenous proteinases that break down seed storage proteins, cereal serpins have been considered as PRs. Protein Z is the most abundant malt and beer protein[8] and has been the first characterized protein in beer. Protein Z with a molecular mass of about 43 kDa is composed of different isoforms with pI in the range of 4.5 – 5.5.[35] Two main subfamilies were recognized, Z4 and Z7 (BSZ4, BSZ7), which are expressed from two related gene families. Protein Z4 is the highly predominant form accounting for approximately 80 % of all protein Z.[5,8] Protein Z4 has also been identified as one of the major beer allergen.[47] It was reported that protein Z4 showed positive correlations with beer foam stability, while protein Z7 showed negative correlation. Moreover, even a trace amount of another subfamily named protein Zx was detected in beer.[48]

Protein Z contains 20 lysines per molecule and about 16 % of the lysine content is glycated during the brewing process through Maillard reaction.[8] The glycated form of protein Z may improve the foam stability and glycation might prevent precipitation of protein Z during the wort boiling step as well. Moreover, protein Z contributes significantly to the lysine content of seed.[5,27]

The proteolytic cleavage of protein Z in the reactive site loop located 37 amino acid residues from the carboxy-terminal was observed. It leads to formation of the C-terminal 363 – 399 fragment with *m/z* 4.03 kDa. The protein chain cleaves probably due to the interaction between protein Z and serine proteases. Cleavage is accompanied by a considerable conformational change forming the heat and protease stable molecule of protein Z that survive the brewing process and is present in beer.[49,50]

2.5.1.3. Non-specific lipid transfer proteins (ns-LTPs)

LTPs constituting the group of PR-14 are ubiquitous plant lipid binding proteins named for their ability to mediate the transport of different classes of lipids between membranes *in vitro*. The biological role of ns-LTPs is still a matter of debate.[5,51] Their role in intracellular lipid transfer is contrasted by findings that LTPs are extracellular secretory proteins. Currently, they are considered to play several *in vivo* roles, including inhibition of growth of bacterial and fungal pathogens, early recognition of pathogens, adaptation of plants to abiotic stress, or inhibition of cysteine proteinases. They are subdivided into two families: ns-LTP1 and ns-LTP2.[5,52,53]

ns-LTP1

Non-specific LTP1, which was initially named probable amylase/protease inhibitor (PAPI), has been studied more thoroughly than ns-LTP2.[51,54] It has been recognized to have a variety of *in vitro* capabilities, besides the transfer of lipids between membranes, also the inhibition of bacterial and fungal pathogens.[55] LTP1 is an abundant protein of the aleurone layers from barley endosperm characterized by a pI of about 9 and consists of 91 amino acid residues for a molecular mass of 9.69 kDa. The protein consists of four α-helices and a C terminal arm (Figure 3), stabilized by four disulfide bonds forming a small hydrophobic cavity between the helices into which a different types of lipids can bind.[35,51,56] LTP1 is glycated during the malting process due to Maillard reactions. Glycation could protect protein from precipitation on unfolding that occurs during wort boiling. Moreover, LTP1 is a highly stable protein that resists temperatures up to 100 °C. This stability may be important for the biological function of LTP1.[51]

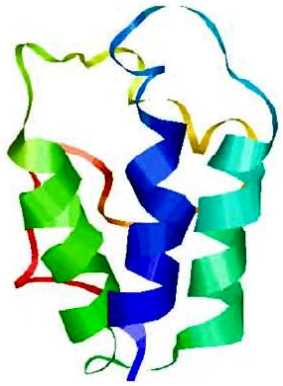


Figure 3: The three dimensional structure of barley seed ns-LTP1[5]

Although LTPs typically bind fatty acids in a non-covalent way, covalently modified forms of LTP1 were also identified.[57] First known modified form of LTP1, named LTP1b, was isolated from barley and beer extract and exhibit a molecular weight 294 Da higher than LTP1.[35] The lipid-like molecule is esterically bound to Asp and was originally described by Lindorff-Larsen et al.[58] as cis-14-hydroxy-10,13-dioxo-7-heptadecenoic acid (CHDH). Nevertheless, later Bakan et al.[57] identified the lipid-like molecule as 9-hydroxy-10-oxo-12(Z)-octadecenoic acid. LTP1b is formed during germination in a physiological process occurring in the endosperm, in contrast with the glycation detected only in the malted samples. According to Žídková et al.[59], this modification can be detected in the MS analysis of intact protein samples or after in-solution enzymatic digestion; however, no lipid-bound peptide was observed in the MS analysis of the in-gel digested LTP1 after sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation because the presence of electrophoretic buffer, denaturing conditions and alkaline pH lead to hydrolysis of the ester bond.[59] LTP1b is stable up to 60 °C, while reducing agents decrease its thermostability and reduced protein denatures in a few hours at the same temperature, as discovered by Matejková et al.[60] In addition, also another isoform of LTP1 named LTP1c was observed in malt by Jégou et al.[54]. This isoform corresponds to a LTP1 protein with two adducts of 294 and 312 Da.[51]

Whereas the native barley seed nsLTP1 does not display any foaming properties, the corresponding beer protein is surface-active. LTP1 becomes a foam-promoting form after unfolding during wort boiling and this improvement is related also to glycation on malting and acylation on mashing. Foam promoting LTP1 form concentrates in beer foam and contributes widely to foam formation.[35,51]

Although modified forms of ns-LTP1 are foam-promoting[52], their increased amount in beer could be disadvantageous as well since they may be responsible for malt derived gushing as mentioned by Hippeli and Elstner.[61] Nevertheless, no apparent correlation has been proved between gushing and nsLTP1 by Hégrová et al.[39] Furthermore, barley LTP1 together with protein Z has been identified as the main beer allergens by Garcia-Casado et al.[47]

ns-LTP2

Non-specific LTP2 has not been studied as deeply as ns-LTP1. In contrast to ns-LTP1 expressed late in grain development and in early stages of germination, ns-LTP2 is expressed during the early stages of grain development.[5] It is a basic protein (pI ~ 8) with a molecular weight of 7.11 kDa consisting of 69 amino acid residues. Ns-LTP2s display low sequence similarity to ns-LTP1s; however, an almost identical proportion of α-helices and random coils in ns-LTP2 and ns-LTP1 suggests that they have a similar fold. Compared to ns-LTP1, ns-LTP2 has higher lipid transfer activity and also is more stable. It is glycated during the malting as well.[35,52] Though similar to ns-LTP1, the role of ns-LTP2 in relation to beer production and quality is unknown at this time. Nevertheless, ns-LTP2 is present in beer and beer foam and its foam-promoting properties may be similar to ns-LTP1.[8] During the malting and brewing process, disulphide bonds in ns-LTP1 and ns-LTP2 are reduced and rearranged resulting in the formation of a dimer composed of ns-LTP1 and ns-LTP2 connected by at least one disulphide bond.[35,52]

2.5.2. Hordeins

Hordeins, alcohol-soluble prolamin fraction of barley proteins, are storage proteins of barley grain and the main protein fraction of barley endosperm. Depending on molecular weights, this protein family can be divided in four general classes:

- γ hordeins – in some literature marked as A hordeins, sulphur-rich proteins of *m/z* less than 20 kDa, not considered as true storage proteins
- B hordeins – sulphur-rich proteins of *m/z* 30 – 45 kDa
- C hordeins – sulphur-poor proteins of *m/z* 49 – 72 kDa
- D hordeins – high molecular weight prolamins with *m/z* of 100 kDa

The B and C hordeins present the major sub-groups accounting for about 70 – 80 % and 10 – 20 %, respectively, while γ and D hordeins are the minor components. Hordeins exist both in monomeric and aggregated forms.[8,9,62]

Hordeins are present in the protein matrix that surrounds the starch granules within the cells of the endosperm. Degradation of the hordein in this matrix during malting is necessary to allow starch degrading enzymes access to the starch, which facilitates complete starch hydrolysis.[63] Influence of hordeins on foaming is not well studied yet.[64] During the brewing process, most of malt hordeins are disappeared. However, hordeins might be involved in beer haze formation, as found by Jin et al.[38] In addition, Šalplachta et al. showed the potential of studying of hordein pattern for the discrimination of barley varieties.[62]

2.1. Protein extraction from plant tissues

Plant protein extraction is the first and crucial step in proteomic studies. Plant tissues contain relatively low levels of proteins whose extraction can be difficult because of the presence of other compounds, such as cell wall and storage polysaccharides, lipids, or phenolic compounds.[23] Moreover, wide range of plant proteins is present and these proteins require specific conditions for their extraction and purification, because their properties vary greatly.[65]

The solubility of plant proteins is closely associated with their intracellular localization.[23] Therefore, extraction and fractionation of plant proteins on the basis of their solubility formed the basis for the first systematic attempt to proteins classification. The classification system of seed proteins is based largely on the work of Osborne[66], who recognized that seed proteins differ in their solubility properties. He defined four groups that were extracted sequentially in water (albumins; comprise mostly enzymatic proteins), dilute salt solutions (globulins; generally occur in protein bodies), alcohol-water mixtures (prolamins; also found in protein bodies as true storage proteins) and dilute acid or alkali (glutelins; probably mainly structural proteins). All these fractions are well known as the "Osborne fractions" or "Osborne groups" and still form the basis for studies of seed proteins.[65,67,68] The term "hordein" became commonly used for the barley prolamin fraction.[69] Terms albumin and globulin have become accepted into the general vocabulary of proteins chemists.[65]

2.2. Protein separation

The analysis of proteins requires methods for the separation of complex protein mixtures into their individual components. Protein separation methods can be both selective and non-selective. Non-selective separation techniques are used for fractionation of complex protein mixture. These methods are based on general protein properties, including their mass, charge, or solubility. By selective method, individual protein can be isolated from a mixture usually by techniques based on very specific protein properties, such as their adsorption characteristics, biological affinities for other molecules, binding specificity or biochemical function. These methods can be used for studying of protein interactions or functions.[1,3]

Several separation techniques are usually used in sequence for protein purification due to the complexity of the protein or peptide mixtures.[1] However, if more separation steps are involved, more proteins can get lost due to technical reasons. Furthermore, the analysis of one complex sample can take quite a long time.

The concentrations of proteins are not evenly distributed within a sample. For all separation methods and for mass spectrometry it is very difficult to detect and analyze very low concentrated proteins in the presence of highly abundant proteins. Moreover, the complex protein samples have often limited stability because many proteins exhibit biological activities and some of them can modify other proteins in the sample mixture.[15]

Many techniques can be used for separation of complex protein mixtures, but not all of these techniques are suitable for proteomics. Major requirements in proteomics include high resolution, high throughput, and the fractionation procedure should be compatible with analysis by mass spectrometry, the major technology platform for protein identification.[3] Before separation, it is necessary to learn as much as possible about the biochemical properties of a protein, such as molecular mass, isoelectric point (pI), solubility properties,

and denaturation temperature, to determine any unusual physical characteristics that will make separation easier.[1] These physical and chemical differences are determined by the number, type, and order of amino acids in the protein, and by post-translational modifications.[3]

According to the biochemical protein properties, protein separation techniques can be divided as follows:

- **Separation by differential solubility characteristics:** Separation by precipitation exploits the differential solubility properties of proteins in solution, determined by the type and charge of amino acids in the molecule.
- **Separation by adsorption**: Separation by adsorption is used in liquid chromatography methods and is based on differential affinity of the protein for the adsorbent or eluting buffer (ion-exchange chromatography, affinity chromatography).
- **Separation by size:** Many techniques use the differential size of individual proteins, including size-exclusion chromatography or SDS-PAGE. Furthermore, dialysis and other membrane processes (ultrafiltration, nanofiltration or reverse osmosis) are used for protein purification and desalting.
- **Separation by electrophoresis:** Electrophoresis is defined as the migration of charged molecules in a solution through an electrical field. Various types of electrophoresis are used for protein separation, including SDS-PAGE, isoelectric focusing or capillary electrophoresis.[1]

The two major groups of techniques in proteomics are gel electrophoresis (GE) and liquid chromatography (LC). They are often used in two-dimensional arrangement where two different fractionation principles are employed one after another.[3]

2.2.1. Separation by electrophoresis

The principle of electrophoresis is the migration of charged particles in an electric field. The rate of migration of charged molecule depends on the strength of the electric field and the charge density of the molecule.[3] The higher the net charge and the smaller the molecule, the faster is its electrophoretic migration. Electrophoretic methods can be applied for the separation of the components of a mixture, but also for creating characteristic images of a sample for differential analysis.

Proteins and peptides are amphoteric substances. They can become positively or negatively charged, depending on their pI and the pH value of the environment. A protein is positively charged if solution pH is below its pI, and negatively charged if solution pH is above its pI. Thus, they will migrate towards the cathode or the anode, respectively. Electrophoretic separations are carried out in buffers with precise pH value and a constant ionic strength.[1,15]

Electrophoresis of protein in the solution is not widely used because the electrophoresis zones are dispersed in the solution, or because the protein mixture will homogenize once the electric field is removed. These effects are minimized, if electrophoresis is carried out in very narrow vessels (capillary electrophoresis) or within a gel matrix (GE), which also allows the fixation of separated proteins in place when the procedure is complete. Gel matrices can be formed in glass tubes or more commonly as slabs between two glass plates. Polyacrylamide gel electrophoresis (PAGE) is one of the most widely used protein separation techniques that facilitate separation by sieving the proteins on the basis of their size. Gels with different pore

sizes can be produced easily and reproducibly by varying the concentration of acrylamide in the polymerization mixture.[1,3]

In non-denaturing or native electrophoresis, proteins are separated in their native form based on charge, size, and shape of the molecule. Non-reduced samples are fractionated for some clinical applications, for instance detection of antibodies. However, denaturing electrophoresis is usually used for protein separation, mainly PAGE with an anionic detergent sodium dodecylsulfate (SDS). SDS-PAGE is the mostly applied electrophoretic method for protein analysis, used for separation of protein subunits based on their size (molecular weight) only, irrespective of charge.[1,15]

2.2.1.1. SDS-PAGE

The basis of the technique is the exposure of proteins to the reducing agent and the detergent SDS in a buffer. Reducing agents, such as mercaptoethanol (ME) or dithiothreitol (DTT), are used to reduce disulfide bonds within cysteines of protein subunit or between subunits. Therefore, proteins become completely unfolded and all quaternary structures are dissolved. Denatured proteins are then stoichiometrically binding to the detergent SDS that carries a large negative charge. SDS masks the charge of the proteins themselves and the formed anionic protein-SDS complexes have a reasonably constant net negative charge per unit mass. Consequently, all proteins including those with basic pIs will migrate towards the anode and therefore, proteins are separated on the polyacrylamide gel according to their molecular weights. The molecular weights of the sample proteins can be estimated with the help of co-migrated standards with known molecular weights, nevertheless SDS-PAGE allows only estimation of molecular mass of a protein and exact masses can be determined with mass spectrometry only. [1,3,15]

In the standard sample preparation procedure for 1D SDS-PAGE electrophoresis, proteins are boiled for approximately 3 minutes in the sample Tris-HCl buffer containing SDS, reducing agent (ME, DTT) and bromophenol blue. For vertical gels, the sample buffer must contain glycerol to prevent mixing of the sample with the upper buffer.[15] Bromophenol blue, so-called tracking dye, migrate ahead of the proteins and is used to monitor the progress of a separation. After an electrophoresis run, the bands on the gels are generally visualized using a protein stain such as Coomassie Brilliant Blue or silver stain.[1] Coomassie blue is the most commonly used protein stain after SDS-PAGE with a sensitivity approximately 100 ng per protein band. Gels are staining in a methanol-acetic acid mixture, used to precipitate the proteins within the gel and preventing them from floating away before analysis. Silver staining increases the level of sensitivity by the ability to detect 1 ng of protein in a band. Silver staining can be used to detection improvement instead of or after Coomassie blue staining.[70]

Polyacrylamide gel

The polyacrylamide gel matrix is formed by polymerizing acrylamide monomers and a small quantity of the cross-linking reagent N,N'-methylenebisacrylamide, in the presence of a catalysts tetramethylethylenediamine (TEMED) and ammonium persulphate as a source of free radicals. This standard catalyst system works only for gels containing neutral and basic buffers. Acidic gels are polymerized with alternative reagents and its preparation is more complicated because polymerization occurs very quickly and is difficult to control. Gels can

be made in the laboratory or purchased precast. Polyacrylamide gels must be polymerized in closed cassettes to exclude oxygen, which would interrupt the polymer chain formation. The polymerization efficiency is also influenced by the monomer concentration, the quality of the reagents, temperature, and pH value. [1,15]

The pore size of the resolving gel is selected based on the molecular mass of the proteins of interest. It is varied by altering the acrylamide concentration (T) and the degree of cross-linking (C).[1] Generally, when T increases, the pore size decreases. In standard gels where T is 15 %, the minimum pore size is achieved when C is approximately 5 %.[3] With higher C values, the gels become brittle and relatively hydrophobic.[15]

Proteins are usually separated on resolving gels that contain 4 – 15 % of acrylamide. Concentration of 15 % T may be used for separation of proteins with molecular mass below 50 kDa. Proteins greater than 500 kDa are often separated on gels with acrylamide concentrations below 7 %. A gradient gel in which the acrylamide concentration increases from top to bottom of the gel is often used to separate a mixture of proteins with a large molecular mass range.[1] Gradients are prepared by continuously changing the acrylamide concentration in the polymerization solution while pouring the gel. The density of the highly concentrated solution is supplemented with glycerol so that the layers in the cassette will not mix.[15]

To improve resolution of proteins within a complex mixture, discontinuous gel matrix is usually used, where a stacking gel is polymerized on the top of the resolving gel. The stacking gel contains a different buffer composition and has larger pore sizes than the resolving gel (usually 3 – 4 % T). As its name implies, it is used to stack or concentrate the proteins into very narrow bands prior to their entry into the resolving gel. Stacking gels are used for 1D separation, when the applied sample molecules are in liquid phase, while for 2D electrophoresis in vertical systems, the stacking gel is not necessary.[1,15]

2.2.1.2. Isoelectric focusing (IEF)

Isoelectric focusing is usually the first dimension separation in 2D GE. In this process, proteins are separated on the basis of their net charge irrespective of their mass. Each protein is specified by pI value determined by the number and type of acidic and basic amino acid residues they contain. IEF is carried out in a pH gradient, allowing each protein to migrate to its isoelectric point, at which its pI value is equivalent to the surrounding pH and its net charge is zero. Therefore, each protein stops in the location of its isoelectric point in the gel. Diffusion still acts against this tendency to focus at a single position, but a protein diffusing away from its isoelectric point becomes charged and therefore moves back towards its focus. Although there may be an initial sieving effect which separates the proteins on the basis of their size, running the gel for a suitably long period of time ensures that all proteins reach their isoelectric points.[3]

IEF can be performed on tube gels or on IPG strip gels that have recently replaced tube gels since they are easier to handle and give more reproducible separations. A pH gradient is formed using ampholytes, which are small polymers containing both positively and negatively charged groups. An ampholyte mixture that exhibits a range of pH values is added to the gel solution prior to polymerization. After the gel formation and current application, the ampholytes migrate to produce the pH gradient.[1,3]

2.2.1.3. Two-dimensional gel electrophoresis (2D GE)

Isoelectric focusing and SDS-PAGE can be combined for separating very complex mixtures of proteins. This technique is called two-dimensional electrophoresis. First, proteins are separated in tube gels, or alternatively on immobilized pH gradient (IPG) strips, by isoelectric focusing according to their charge. The tube gel containing the separated proteins is then placed on top of an SDS-PAGE slab gel, and proteins are separated according to their mass. Contemporary standard 2D GE systems are capable of resolving approximately 2500 protein spots.[1,3]

2.2.2. Liquid chromatography

Any separation technique that distributes the components of a mixture between two phases, a fixed stationary phase and a free-moving mobile phase, is known as chromatography. As the mobile phase moves over the stationary phase, the components of the mixture can interact with the molecules of both phases. Molecules with the lowest affinity for the stationary phase will move the most quickly because they tend to remain in the solvent, while molecules with the highest affinity move the most slowly because they tend to stay associated with the stationary phase and are left behind. This results in the mixture partition into a series of fractions, which can be eluted and collected individually.

In proteomics, LC is used more often than other chromatographic formats because of its versatility and compatibility with mass spectrometry. Contrary to gel electrophoresis, liquid chromatography is suitable for the separation of both proteins and peptides. Various LC methods can exploit different separation principles, such as size, charge, hydrophobicity and affinity for particular ligands. As well as is the case of electrophoresis, the highest-resolution separations are achieved when two or more separation principles are applied one after another in two-dimensional arrangement.

Liquid chromatographs are often connected with UV (ultraviolet) detectors. Proteins are measured traditionally at 280 nm. At this wavelength, aromatic amino (primarily tryptophan and tyrosine) absorb best so proteins with few of these amino acids may not absorb as strongly as expected. Alternatively, the UV light at a wavelength of 214 nm is absorbed by the peptide bond and this detection yields much more sensitivity than 280 nm. Otherwise, chromatographs can be linked directly to electrospray ionization (ESI) mass spectrometers (LC-MS or LC-MS/MS) for fully automatic peptide separation and analysis.[1,3,71]

2.2.2.1. Reversed phase chromatography

Reversed phase separations are usually carried out using high performance liquid chromatography (RP HPLC). Reversed phase resin consists of hydrophobic ligands (such as C4 to C18 alkyl groups), so proteins (peptides) are separated according to their hydrophobicity. However, RP HPLC results in a quasi-mass-dependent separation because retention of molecules tends to increase with their molecular mass. Separation is performed in gradient elution that is achieved by gradually increasing the amount of an organic modifier in the elution buffer, which disrupts the hydrophobic interactions of proteins on the RP resin. Of all the chromatography techniques used in proteomics, RP HPLC is the most powerful method and provides the highest resolution.[3]

2.2.2.2. Size exclusion chromatography

Size exclusion chromatography (SEC), also known as gel filtration chromatography, is used for separation of proteins (or peptides) according to their size. The column is packed with inert beads made of a porous compound such as agarose because this separative principle does not require any chemical interaction between the solutes and the stationary phase. Molecules larger than the pores in the beads are excluded, moving quickly through the column and eluting from the column in the shortest times. Commercial columns are able to separate protein or peptide mixtures in different size ranges. [1,3]

2.2.2.3. Affinity chromatography

Affinity chromatography (AC) is based on the reversible interactions between the target molecules and the specific ligands immobilized on a stationary phase. The process usually consists of three steps: binding, washing and eluting. In the binding step, samples containing the target molecules are loaded onto the column and allowed to bind. In the washing step, un-bound components are washed off the column, while the target bound molecules are retained. In the eluting step, specific components in an eluting solution are used to break the target-ligand interactions, and consequently, the target molecules are eluted.[72]

Several types of AC can be applied in proteomic studies. For instance, beads containing antibodies can be used to isolate a single protein or peptide from a complex mixture, and lectin affinity chromatography is widely used for glycoprotein or glycopeptide purification.[3]

2.3. Protein identification by mass spectrometry

Although several technologies have been developed for protein identification, current proteomic analysis would be impossible without recent advances in mass spectrometry. A mass spectrometer is an instrument that can measure the mass-to-charge ratio (*m/z*) of ions based on their motion in an electric or magnetic field. Sample molecules are converted into ions (positive or negative) in the gas phase and separated in a vacuum according to their *m/z* ratio. Molecular masses can be determined with a high degree of accuracy, allowing the determination of the sample molecular composition.

Table 3: Three components of a mass spectrometer used in proteomics.[15]

Ionization	**Separation**	**Detection**
Ionization source	Analyzer	Detector
MALDI	Time-of-flight (TOF)	
ESI	TOF/TOF	
	Quadrupole	
	Ion trap	
	Orbitrap	
	Hybrid combinations	

Mass spectrometer typically consists of three components (Table 3):

- source of ions – in a vacuum convert the analyte into gas phase ions which are accelerated in an electric field towards analyzer;
- mass analyzer – separates ions according to their *m/z* ratios on their way to the detector;
- ion detector – records the impact of individual ions.[3,15]

The analysis of proteins and other macromolecules by mass spectrometry has been always difficult since larger molecules are broken up by the volatization and ionization process producing a collection of random fragments. It has changed in the nineties with the development of so-called soft-ionization methods that allow the ionization and detection of large, non-volatile and labile molecules by mass spectrometry. In proteomics, two soft-ionization methods are widely used:[3,14]

- **matrix-assisted laser desorption/ionization (MALDI)** – will be described in the next chapter (2.3.1);
- **electrospray ionization (ESI)** – in this method, the analyte is dissolved and forced through a narrow needle held at high voltage. From the needle arises a fine spray of charged droplets that is directed into the vacuum chamber of the mass spectrometer. The droplets are then dried using a stream of inert gas, resulting in gas-phase ions that are accelerated through the analyzer towards the detector.

MALDI is predominantly used for the analysis of simple peptide mixtures, such as the peptides derived from a single spot from a SDS-gel, whereas ESI is suitable for the analysis of complex mixtures. Moreover, ESI is often connected with liquid chromatograph because gas-phase ions are produced from solution in this technique.[3] MALDI instrument can not be directly coupled to the liquid chromatographic techniques, but the LC-separated peptide or protein fractions can be spotted onto the MALDI target, and then analyzed in an "offline" approach.

While ESI produces mainly multiply protonated peptide ions $[M + nH]^{n+}$, MALDI generates mainly singly protonated peptide ions $[M + H]^{+}$, although multiply charged species are also sometimes observed. In the case of proteins, both singly and multiply charged ions can be generated in MALDI.[14]

2.3.1. MALDI-TOF/TOF instrumentation

MALDI-TOF/TOF is a very important proteomic tool. This configuration supports both MS and tandem MS/MS measurements in a single system.[15]

2.3.1.1. MALDI ionization

Before the analysis, the analyte is mixed with a large excess of an aromatic compound called matrix that can absorb light at the wavelength of the laser used with the mass spectrometer. The most commonly used matrices in proteomics are described in the chapter 2.3.2.1. The analyte and matrix are dissolved in an organic solvent and placed on a metallic target (i.e. MALDI plate). The solvent evaporates, thus the analyte becomes incorporated into the crystal lattice of the matrix. The MALDI plate is then placed in the vacuum chamber of the mass spectrometer and a high voltage is applied. The crystals are targeted with a short laser pulse and the laser energy is absorbed by the crystals, which causes desorption and ionization of the matrix and analyte, either by protonation (positively charged ions) or

by deprotonation (negatively charged ions). The ions are then accelerated into the MS analyzer. Due to the pulsed nature of MALDI, it has been predominantly coupled with the time-of-flight (TOF) analyzer or configurations thereof.[3,15]

2.3.1.2. Time-of-flight (TOF) analyzer

TOF analyzer is a pulsed analyzer routinely coupled with a MALDI ion source. No electric field is required for separation and mass measurement is determined by measuring the time-of-flight of an ion in the analyzer region. The smaller the molecule, the faster it will travel the distance of the flight tube towards the detector. [3,15]

The linear TOF analyzer is the simplest form and exhibits the lower performance with respect to resolution and mass accuracy. It is shown in Figure 4 (the linear detector without the collision cell connection). The mass accuracy of a linear TOF instrument is poor. The performance of TOF analyzers was therefore improved by two developments: delayed extraction of ions from the source for a short period of time, and incorporation of a reflectron.[15]

The reflectron (or ion mirror) was designed to re-focus the ions onto the detector.[14] It is shown in Figure 4. Reflectron improves resolution in two ways:

- by acting as an ion mirror reversing the trajectory of the ions in the flight tube and effectively increasing the length of the flight tube;
- by reducing an ion's kinetic energy spread.

Ions of the same mass formed in the source can have different kinetic energies when leaving the source. It depends on their position in the source when the accelerating voltage is applied. Consequently, ions of the same mass arrive at the detector at different times, thus reducing resolution and mass accuracy. A reflectron can accommodate these small differences in kinetic energy and greatly improve resolution and subsequently mass accuracy. Reflectrons are incorporated as standard in most commercial TOF mass spectrometers.[15]

2.3.1.3. TOF/TOF analyzer

In tandem mass spectrometry (or MS/MS), selected ions of individual *m/z* values are subjected to fragmentation. The first TOF analyzer selects the precursor ion, high energy collisions then occur within the collision cell, and the second TOF analyzer resolves the ions (Figure 4). Fragmentation is typically performed by collision induced dissociation (CID), a mechanism of molecular ions fragmenting in the gas phase. Peptide molecular ions are allowed to collide within a cell with neutral gas molecules (helium, nitrogen or argon). Some of the kinetic energy possessed by the molecular ion is converted into internal energy, which results in bond breakage and the fragmentation of the molecular ion into smaller fragments.

A TOF/TOF analyzer coupled with a MALDI ion source enables the generation of PMF data and peptide sequence data in a single instrument. The configuration achieves high sensitivity and high resolution in both MS and MS/MS modes.[15]

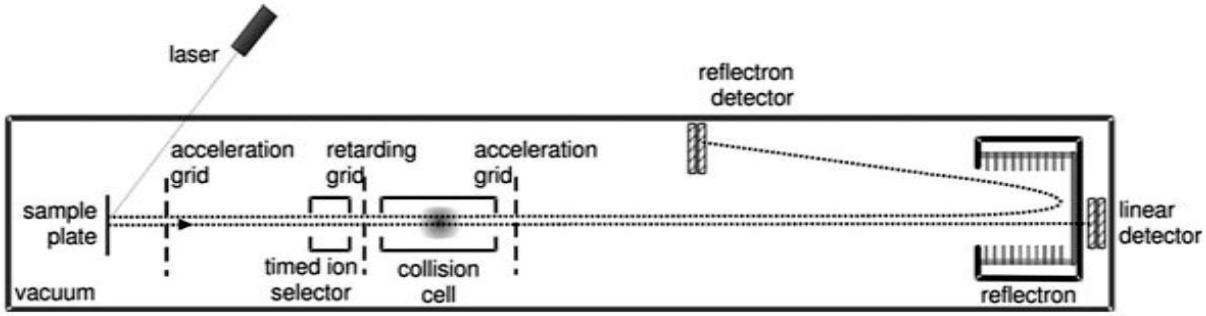


Figure 4: Schema of TOF/TOF analyzer[15]

2.3.2. Applications of MALDI-TOF MS in proteomics

In proteome studies, highly complex mixture of proteins is usually analyzed. Therefore, one or combination of several separation steps of proteins or peptides (mentioned in the chapter 2.2) are required prior to MALDI-TOF MS analysis.[14] For successful protein identification, fractionated proteins are digested into peptides, most frequently using proteolytic enzymes. Then, peptides are purified and spotted with a suitable matrix on a MALDI target for further analysis by MALDI-TOF MS.[3]

2.3.2.1. Sample preparation for proteomic analysis by MALDI-TOF MS

Sample preparation is a crucial procedure in MALDI-TOF MS analysis of peptides and proteins. Protein or peptide samples need to be purified prior to MS analysis. If possible, volatile buffers, such as ammonium bicarbonate, should be used in the final stages of purification of proteins.[14,15]

Protein cleavage

The analysis of proteins by MALDI-TOF MS peptide mass mapping involves proteolytic degradation of proteins into peptides. Prior to proteolytic digestion, it is convenient to reduce the S–S bridges and S-alkylate the cysteine residues of proteins. This blocks their chemical reactivity and secondary structure formation, thus it increases the accessibility to digesting proteases and improves the detection efficiency of Cys-containing peptides in MALDI-TOF MS. Iodoacetamide and 4-vinylpyridine are good S-alkylating reagents.

Trypsin presents the most often used proteolytic reagent. It is a highly active and specific protease, cleaving from C-terminal to Lys and Arg residues, to generate peptides within the mass range from 500 to 5000 Da that are suitable for PMF. Other enzymes suitable for protein proteolysis are endoproteases Lys-C, Asp-N and Glu-C. Chymotrypsin, proteinase K, and subtilisin are less-specific proteases which are helpful for proteolytic cleavage of very compact or stable protein structures.[14] Chymotrypsin is frequently used as an alternative to trypsin, for example for studying of protein glycations. Since glycation occurring mostly at lysine, treatment with trypsin would lead to formation of relatively long glycated peptides that are more difficult for MS/MS fragmentation, as mentioned by Petry-Podgórska et al.[73]

The proteolytic digest can be performed either in-solution or in-gel. For proteins separated on gel, the digestion is frequently performed while still in the gel, and subsequently, resulting peptides are extracted into the surrounding solvent by extraction procedure using acetonitrile and trifluoroacetic acid (TFA).[14]

Chemical methods of protein cleavage are possible as well, but are less commonly used. They are often used for specific applications, where the enzymatic proteolysis is not suitable.[14,15]

Sample purification

Desalting and concentrating of protein or peptide samples are appropriate prior to MALDI-TOF MS analysis. It is also important to remove all contaminants, such as ionic detergents (SDS), which could interfere with the formation of analyte-matrix crystals during sample preparation. Especially if only small amount of material are available, miniaturized solid-phase extraction (SPE) methods are recommended. Miniaturized SPE columns can be purchased ready-made, or custom-made using Eppendorff GELoader tips packed with proper resin. Reversed phase C18 resin is suitable for protein or peptide purification, whereas phosphopeptides are retained by IMAC or TiO_2, and glycopeptides by graphite or HILIC.[14,15]

MALDI matrices

Before mixing the protein or peptide sample with MALDI matrix, the pH should be adjusted to less than 3 by addition of TFA to obtain a good crystallization of matrix and analyte.[14] The most commonly used matrices in proteomics are:

- α-cyano-4-hydroxycinnamic acid (CHCA) is typically the standard matrix for a tryptic digest and affords high sensitivity for the detection of peptides;
- 2,5-dihydoxybenzoic acid (DHB) enables peptide analysis as well as detection of proteins and the analysis of oligosaccharides released from glycoproteins;
- sinapinic acid (SA, 4-hydroxy-3,5-dimethoxycinnamic acid) is suitable for analysis of proteins and large polypeptides.[15]

The quality of measured MALDI-TOF mass spectra are significantly influenced by many factors, including choice of suitable matrix, matrix and sample concentrations and matrix/sample ratio, pH adjustment crystallization conditions, or the use of additives.[74,75]

2.3.2.2. Peptide mass mapping

MALDI-TOF MS analysis of a digested protein provides a spectrum of peptide peaks, thus a list of peptide mass called fingerprint. For the protein identification, individual peak masses are compared with theoretical peptide masses of considered protein in a database, calculated from theoretical cleavage with used enzyme (Figure 5). This type of MS-based protein identification strategy is known as peptide mass fingerprinting (PMF). MS analysis of peptides derived from a protein by enzymatic digestion also reveals the presence of a chemical modification by a mass increment or a mass deficit relative to the expected masses of the unmodified peptides.[14] However, MALDI-TOF MS PMF is not a suitable method for protein identification when a complex mixture is under investigation. In this case, tandem mass spectrometry MS/MS is almost exclusively required.[15]

The PMF technique is very robust because the masses of intact peptides are extremely discriminatory. Nevertheless, the probability of finding a matching protein depends on:

- the quality of the experimental data, involving the quality and relative intensity of the peaks in the mass spectrum, the mass accuracy of the instrument, the coverage of the protein, and possible interfering factors such as post-translational modifications and mis-cleavages;
- the availability of sequence information for the organism from which the experimental sample was obtained.[3]

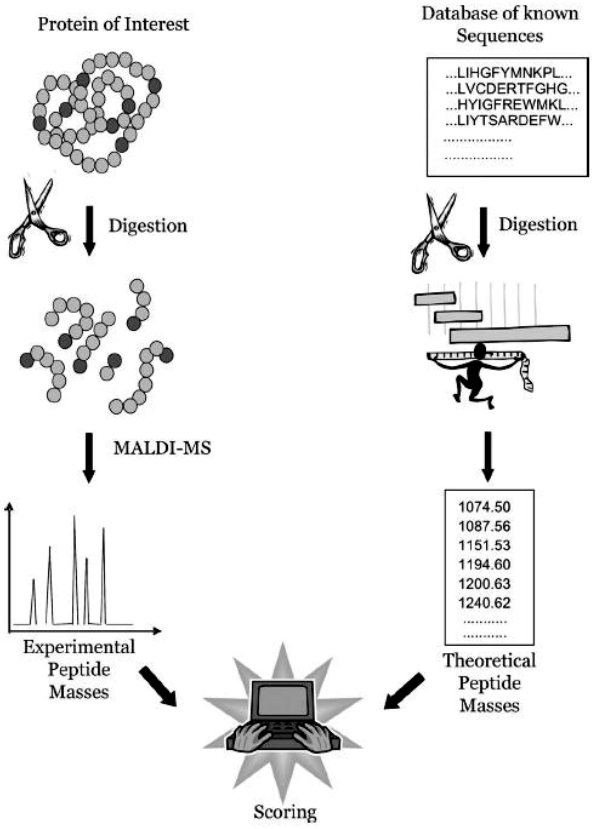


Figure 5: Principle of protein identification by MALDI-TOF MS PMF.[14]

HPLC separation of peptides is sometimes necessary prior to MALDI-TOF MS for detection and characterization of all peptides in a mixture. In theory, all peptides should be present in equal concentration in a protease digest. However, this is not reflected in MALDI-TOF spectra for several reasons. The ionization efficiency of different peptides can vary and certain peptides are not detected when present in a peptide mix. As an example, arginine-containing peptides are found to ionize better than arginine-deficient peptides.[14]

2.3.2.3. Tandem mass spectrometry (MS/MS)

Where peptide mass fingerprinting is insufficient for identification of any proteins present in a given sample, tandem mass spectrometry (MS/MS or analysis of fragmentation ions) can be applied. The fragment CID spectrum of one or more individual peptides may provide important additional information.

These data can be used in following ways:

- protein identification – correlative database searching of proteins whose peptides would likely yield similar CID spectra under the same fragmentation conditions;
- deducing partial *de-novo* peptide sequences by interpretation of the peaks of the mass spectrum (manually or automatically). Acquired sequences can be consequently searched in database. The advantage of both these approaches is that correlative searching is not limited to databases of full protein sequences;
- detection and localization of post-translational modifications.[3,14]

2.3.2.4. Peptide Sequencing by MALDI-TOF MS/MS

Traditionally, proteins were characterized by *de-novo* sequencing using automated Edman degradation and amino acid composition analysis. Currently, these techniques tend to be replaced by MS which provides more flexibility and sensitivity and is applicable to the analysis of protein and peptide mixtures. MS/MS is very powerful for peptide characterization and identification via sequencing and sequence database searching.

Since the fragmentation produces various set of ions, the interpretation of CID spectra is difficult. As mentioned previously, MALDI produces mainly singly protonated peptide ions $[M + H]^+$. Fragments resulting from low-energy CID are shown in Figure 6. The most efficient is the cleavage at the amide bond that results in y- and b-ions (according to the Roepstorff/Fohlman nomenclature), depending on which part of the peptide will retain the charge:

- y_n-ions – the charge is retained on the C-terminal fragment (n is the number of residues in the fragment) and y_n-ions will contain the C-terminus of the peptide and extensions from this residue;
- b_n-ions – the charge is retained on the N-terminal fragment and b_n-ions will contain the N-terminal amino acid and extensions from this residue.[14,15]

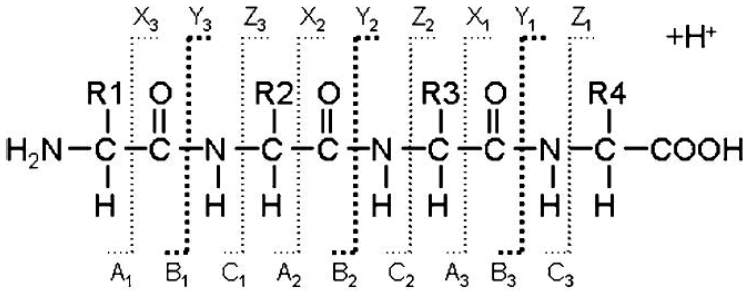


Figure 6: Nomenclature for peptide sequencing. Fragments resulting from backbone cleavages are mainly observed when MALDI peptide ions are fragmented in low-energy CID tandem mass spectrometers.[14]

The mass difference of y_n and y_{n+1} corresponds to the mass of the amino acid residues and can therefore be used for peptide sequence deducing. Upon low-energy CID of the peptides, the dominating ions-series will be a, b and y fragments.[14] The masses of individual amino acid residues and their single letter abbreviations used in sequencing are shown in the chapter 2.1 in the Table 1. From this table it is evident that two pairs of residues have very similar (Gln and Lys) or identical masses (Leu and Ile).[3] The isobaric residues Leu and Ile can be differentiating at higher collision energies, when the peptide is additionally fragmented at

the amino acid side-chains.[15] Moreover, multiple breakages producing internal fragments of several contiguous amino acids as well as immonium ions representing single amino acids may also be observed in the fragment spectrum.[3]

2.3.2.5. Protein quantification using mass spectrometry

Mass spectrometry can be also used for relative of absolute protein quantification that can be performed using stable isotope labelling or by a non-labelling software approach. Stable isotope labelling has been used in mass spectrometry for a long time. Chemically identical analytes with a different stable isotope composition can be separated using mass spectrometry due to the mass difference associated with the different isotopes. The ratio of the different analytes signal intensity is indicative of their relative abundance. The most common methods involve incorporation of stable isotopes into the protein/peptide analyte using isotope coded affinity tags (ICAT™), isobaric tags for relative and absolute quantification (iTRAQ), and stable isotope labelling with amino acids in cell culture (SILAC). While ICAT™ and SILAC measure the relative abundance in MS mode, iTRAQ measures the relative abundance in MS/MS product ion mode.

The iTRAQ reagents are designed to allow simultaneous labelling of up to four samples with four reagents of the same mass (isobaric). The iTRAQ reagent consists of three components:

- a reporter group (with four different masses);
- a balance group (for balancing the reporter group mass);
- N-hydroxysuccinimide ester group that reacts with primary amines at the peptide amino-termini and lysine side-chains.

Fragmentation of these four tags in MS/MS produces four reporter ions (*m/z* 114.1, 115.1, 116.1, 117.1 Da) which are used for quantification of the four samples.[15]

2.4. Analysis of protein post-translational modifications (modificomics)

The comprehensive analysis of post-translational modifications is essential for a true understanding of a cell's biology.[16] Modificomics is the most frequently studied area of interest in proteomics research since PTM information cannot be determined at the DNA level, but is probably also the most difficult.[15,20] Each protein could potentially be modified in different ways; however, it is not necessary that such a modification will take place. Most PTMs are therefore discovered accidentally when individual proteins, complexes or pathways are studied.

Formerly, the analysis of post-translational modifications at the proteomic level was limited because of the lack of suitable methods.[3] Theoretically, it can be simple to localize the modifications by measurements of increase or decrease the molecular mass. Still, there are many technical challenges such as ion suppression, purity and instability of the modified peptides, sequence coverage, and quantification that together make the mapping of PTMs difficult.[76] However, many of the techniques can now be adapted for this type of experiment.[3] The development of chemical tagging and enrichment methods, in combination with an advance in MS instrumentation, would improve the sensitivity and accuracy for the determination of PTMs. In contrast to the progress made in animal and human modificomics, there have been relatively few studies in plant modificomics.[76]

The key issue in PTM analysis is the enrichment of the modified protein or peptide from a complex mixture because modified proteins are often expressed with low abundance[15] and without enrichment, mass spectrometric analysis has low efficiency to detect PTM proteins or peptides. The most commonly used enrichment methods include: affinity enrichment (e.g. lectin affinity used for protein glycosylation, antibody-based western blot analysis), chemical derivatization or ionic interaction-based enrichment (e.g. immobilized metal-affinity chromatography IMAC and titanium dioxide used for protein phosphorylation).[17]

2.4.1. Analysis of *N*-glycoproteins

Glycoproteomics is a relatively new sub-discipline of proteomics and the technology for glycoprotein characterization is still in the early stages of development.[3] The analysis of the whole glycoproteome of a plant extract involves the determination of:

- which genes encode the glycoproteins;
- which sites of the potential *N*-glycosylation sites are actually glycosylated;
- structure of the attached glycans.

Such identifications have already been realized in animal cells, while plant glycoproteome is examined to a lesser extent.[23]

Conventional glycoanalytic techniques are laborious and time-consuming because multiple steps are necessary. The enrichment of glycoproteins (or glycopeptides after digestion) from a complex mixture is required. The most common approach in characterizing *N*-linked glycosylation involves the release of glycans from the isolated glycoprotein because direct and simultaneous analysis of both the protein and glycan part is difficult. Proteins with relatively simple oligosaccharide chain can be easily studied by matrix-assisted laser desorption/ionization MALDI-TOF MS, for example peptides with mass shifts of + 162 Da or multiples thereof that are indicative of glycated proteins modified with hexose sugars. However, large *N*-glycan-substituted peptides are difficult to study by MALDI-TOF MS because of their large mass falling outside the optimal detection limit, and their heterogeneous nature resulting in broad peaks.[3,77] Glycopeptides also often exhibit poor ionization efficiency in comparison to non-glycosylated peptides, leading to suppressed signals and decreased detection sensitivity. Therefore, glycoproteins needs to be digested using some non-specific protease generating shorter fragments, and separated from non-glycosylated peptides via chromatographic or other purification methods.[77]

2.4.1.1. Lectin affinity enrichment

Glycoprotein enrichment is generally carried out by lectin-affinity chromatography. Lectins are plant proteins able to specifically bind oligosaccharide moieties.[3] Lectin-carbohydrate interactions are mainly reversible and can be inhibited by the inhibiting sugar. This makes lectins ideal candidates as affinity ligands in affinity purification of glycoconjugates.[72] Many lectins are available that have very specific ligands, but only a few of them are available for the detection of plant glycans. The summary of lectins used for plant glycans enrichment is present in the Table 4.[3,23]

Table 4: Lectins used for plant glycans enrichment.[23]

lectin	specificity	sugar inhibitor
concanavalin A (ConA) from *Canavalia ensiformis*	α-D-mannose α-D-glucose	methyl α-D-mannopyranoside
Galanthus nivalis agglutinin (GNA)	terminal α1-3 mannose	methyl α-D-mannopyranoside
wheat germ agglutinin (WGA) from *Triticum aestivum*	terminal GlcNAc internal chitobiose units	GlcNAc chitotriose, chitobiose

Concanavalin A (ConA), one of the most well characterized and widely used lectins, is derived from *Canavalia ensiformis* (Jack bean) seeds. It binds to α-mannose, and to α-glucose with weaker affinity. At neutral and alkaline pH, ConA exists as a tetramer of four identical subunits (Figure 7), while below pH 5.6, ConA dissociates into dimers. Divalent metal ions such as calcium (Ca^{2+}) or magnesium (Mg^{2+}) need to be present to keep ConA active for its binding to carbohydrates.

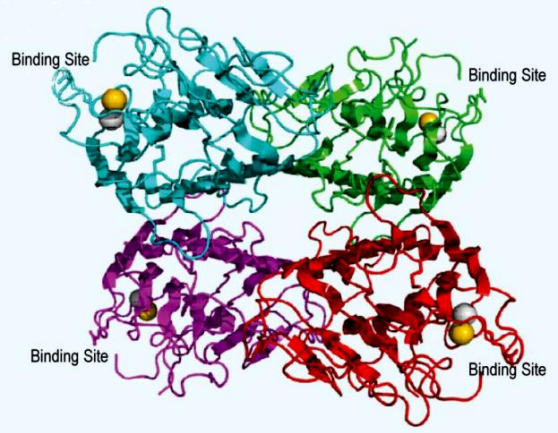


Figure 7: Crystallographic structure of ConA - the four monomer units, while each of them can bind calcium and manganese ions.[72]

Usually high-mannose type *N*-glycans bind to ConA strongly, and some hybrid type glycans can bind to ConA with high affinity as well, while complex type glycans usually have very weak affinity towards ConA.[72] In addition to glycoprotein enrichment, lectin-affinity chromatography is also useful for the purification of glycopeptides following proteolytic digestion, or for the purification of glycans.[3]

2.4.1.2. Deglycosylation and glycan analysis

Releasing of glycan from a glycoprotein or glycopeptide (i.e. deglycosylation) can be achieved in a single step using several enzymes. Endoglycosidase H (Endo H) is able to release high-mannose type *N*-glycans only. Endo H hydrolyzes the glycosidic bond between the two GlcNAc residues on the core of the *N*-glycan. Peptide-*N*-glycosidases (PNGases), PNGase F and PNGase A, hydrolyze the bond between the Asn of the peptide backbone and the proximal GlcNAc of the oligosaccharide part, thus the Asn residue is deamidated to Asp. Since the deglycosylated peptide mass is only 1 Da higher compared to the predicted mass of the unmodified peptide, this treatment is useful for mass spectrometry glycoprotein identification. PNGases have specific activity for both high-mannose type and

complex type *N*-glycans. Nevertheless, PNGase F does not release *N*-glycans containing α1-3 fucose residue linked to the proximal GlcNAc, thus the alternative PNGase A must be used. Nevertheless, PNGase A is almost only efficient on glycopeptides and therefore requires the proteolytic digestion of the glycoprotein prior to deglycosylation.[3,23]

There are several studies published, where the in-gel or in-solution deglycosylation of proteins or peptides were used. For instance, Songsrirote et al.[78] and Küster et al.[79] successfully performed the in-gel protein deglycosylation. Songsrirote et al.[78], Yu et al.[80], Laštovičková et. al[81], Küster et al.[79] or Devakumar et al.[82] performed the in-solution deglycosylation of glycoproteins and Liu et al.[83] performed the in-solution of glycopeptides after trypsin digestion.

2.4.1.3. Glycopeptide analysis

The direct analysis of intact glycopeptides by mass spectrometry offers sequence information on both peptide and glycan moiety, and the detailed knowledge of protein glycosylation at the proteomescale is becoming an important aspect of post-genomic research. However, this method has so far been less commonly used. The analysis of glycopeptides after proteolytic digest without any pretreatment is difficult because non-glycosylated peptides interfere with ionization of glycopeptides and cause the considerable ion suppression. Moreover, several different glycoforms (one glycosylation site carries a multitude of glycans) can be present in a relatively low concentration in the total peptide pool. Therefore, the removing of non-glycopeptides from the proteolytic digest is necessary for efficient analysis.

Lectin-affinity chromatography is often used to glycopeptides enrichment with the advantage of the relatively broad specificity of lectins. Alternatively, approaches based on general physical and chemical properties of glycopeptides are valuable. Glycopeptides are in general more hydrophilic than non-glycosylated peptides, therefore, the enrichment method based on hydrophilic interaction with matrices such as cellulose or Sepharose, or hydrophilic interaction-liquid chromatography (HILIC; or normal phase-chromatography) can be performed. In addition, glycopeptides can be significantly enriched by size exclusion chromatography because most tryptic glycopeptides in a complex peptide/glycopeptide mixture have a relatively high mass. Glycopeptides are often purified on porous graphitized carbon resin; however, this stationary phase is not suitable for selective capturing of glycopeptides from proteolytic digests because it binds strongly to non-glycosyated peptides.[84,85]

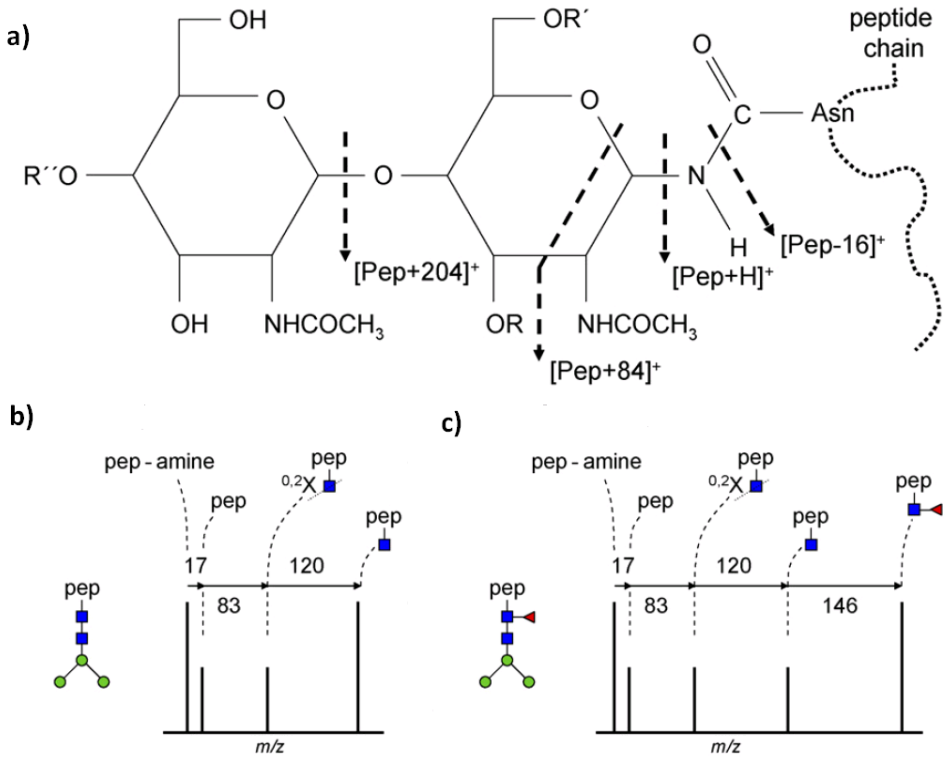


Figure 8: Positive-mode MALDI–TOF/TOF MS fragmentation of glycopeptides; a) schematic representation of the fragmentations occurring near the innermost GlcNAc with retention of the intact peptide moiety; b) characteristic fragment pattern for non-core-fucosylated N-glycopeptides, and c) core-fucosylated N-glycopeptides.[84]

MALDI-TOF/TOF MS of *N*-glycopeptides results in following fragment ion signals (Figure 8):

- $[M_{pep} + H - 17]^+$ - prominent signal that arises from the cleavage of the side-chain amide bond of the glycosylated asparagine
- $[M_{pep} + H]^+$
- $[M_{pep} + H + 83]^+$ - a $^{0,2}X$-ring cleavage of the innermost *N*-acetylglucosamine
- $[M_{pep} + H + 203]^+$ - Y-type cleavage of the chitobiose core in case of a non-fucosylated core
- $[M_{pep} + H + 349]^+$ - Y-type cleavage of the chitobiose core in case of a monofucosylated core
- peptide bond cleavages - predominantly y-type and b-type fragment ion signals, occasionally deamination or elimination of water

Glycopeptide-marker ions in CID spectra are usually low-molecular-weight oxonium ions of *m/z* 204 (GlcNAc), *m/z* 186 or *m/z* 168 (elimination of 1 or 2 water molecules from the GlcNAc oxonium ion), and *m/z* 366 ($Man_1GlcNAc_1$), among others.[84]

3. AIMS OF THE THESIS

The main goal of the thesis is to investigate barley proteins and their changes that occur during the malting and brewing process. In addition, attention is paid to barley protein post-translational modifications, namely enzymatic *N*-glycosylations and non-enzymatic glycations. The particular aims are formulated as follows:

- The identification of water-soluble proteins by MALDI-TOF MS in barley grain and malt and investigation of the protein changes during individual steps of the malting and brewing process using various proteomic separation techniques.
- The monitoring of low-molecular weight water-soluble proteins and their changes.
- The comparison of various barley varieties, including those allowed for the Czech beer production, variety with well-proven malting properties, as well as one non-malting variety.
- The analysis of barley alcohol-soluble proteins (hordeins) and study of their changes during malting.
- The investigation of quantitative changes of selected proteins during malting using the iTRAQ method.
- The investigation of non-enzymatic glycation of barley water-soluble proteins formation during malting.
- The analysis of glycosylated barley water-soluble proteins. The identification of these minor proteins after their enrichment using the ConA affinity chromatography.
- The optimization of analysis of the oligosaccharide part of glycosylated proteins.

4. EXPERIMENTAL

4.1. Chemicals

All chemicals used were purchased through the corporation Sigma-Aldrich (Schnelldorf, Germany) or Fluka (Buchs, Switzerland), unless otherwise stated. Acrylamide, SDS, molecular weight standard (broad range) for SDS-PAGE, Laemmli Sample Buffer, Bio-Safe Coomassie, and ReadyPrep 2D Starter Kit were obtained from Bio-Rad (Philadelphia, PA, USA). Trypsin and chymotrypsin (sequencing grade from bovine pancreas) were obtained from Roche Diagnostics (Manheim, Germany). MALDI matrices, namely CHCA, DHB and SA were obtained from LaserBio Labs (Sophia-Antipolis Cedex, France).

4.2. Samples

Barley grain, grain from 1st to 5th day of malting, green malt and malt, as well as sweet wort, wort and green beer samples, were obtained from RIBM, Brno, CZ. Malt was prepared according to the micromalting laboratory method traditionally used in the RIBM[86]. Barley was steeped and germinated for 144 h at 14.5 °C. Aliquots were collected each 24 hours during the malting and dried for 4 h at 80 °C to prevent biochemical changes. Green malt was dried for 22 h at the temperature increasing from 50 °C to 80 °C. The grain samples were milled in a rotating grinder and obtained flour was used for protein extraction. Sweet wort, wort and green beer liquid samples were freeze dried for further analysis.

Grain and malt of six spring 2-row certified barley varieties were investigated (Table 5). Jersey is a representative of a very good malting barley variety, recommended for malt export. Tolar, Blaník, Bojos and Malz are among varieties allowed for production of beer labelled with PGI Czech beer. AF Lucius is a non-malting hull-less variety grown at the Experimental Station of Mendel University in Žabčice near Brno (CZ). The name AF Lucius was registered in 2009, before that, this variety was known as experimental line KM 1910.[87]

Table 5: Summary of studied barley varieties[87]

Barley variety	Country of origin	Year of registration
Jersey	NL	2000
Tolar	CZ	1997
Malz	CZ	2002
Bojos	CZ	2005
Blaník	NL	2007
AF Lucius	CZ	2009

4.3. Protein extraction

Proteins were extracted from 50 mg of milled barley sample twice with 0.5 mL of suitable solvent (or in larger quantities in the same sample-to-solvent ratio). Protein albumin fraction was extracted with deionized water, and alcohol-soluble prolamin fraction (hordeins) with 60% ethanol and 2% DTT. Extractions were carried out in the shaker for 30 min at the room temperature. The mixtures were centrifuged in MiniSpin plus centrifuge (Eppendorf, Hamburg, Germany) at 14,000 rpm for 10 minutes and the two supernatants from one sample were combined. Water-soluble extracts were lyophilized, whereas alcohol-soluble extracts were dried down in SpeedVac Concentrator 5301 (Eppendorf).

4.4. Protein separation

4.4.1. Electrophoretic separations

4.4.1.1. 1D GE – SDS-PAGE

Proteins were separated using the Mini-PROTEAN system (Bio-Rad) on 10-well gels, either on precast Tris-HCl linear gradient polyacrylamide gels 4 – 20 % (Bio-Rad), or on manually prepared linear gels (with 12 or 15 % of acrylamide). The resolving gel was prepared using following solutions: acrylamide/bisacrylamide solution, 10% SDS, 750 mM Tris-HCl buffer (pH 8.8), 10% ammonium persulphate and TEMED. After resolving gel polymerization, the focusing gel was prepared using the same solutions, only in the buffer of different pH (250 mM Tris-HCl, pH 6.8). The acrylamide concentration of 3 % was used. A comb was inserted on the top of the focusing gel to create the sample wells.

For gel electrophoresis, protein lyophilized extracts were dissolved in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2 % SDS, 25 % glycerol, 0.01 % Bromophenol Blue; mixed with β-mercaptoethanol in the ratio 15:1) and boiled for five minutes in a water bath. For example, lyophilized extract from 50 mg of milled barley sample (approximately 4 mg) was dissolved in 200 μL of sample buffer. Subsequently, individual samples were loaded on the gel in each comb (usually 8 – 12 μL). Protein standard solution (broad range, Bio-Rad) was loaded in at least one comb. Separation was performed in running buffer (composed of 0.025 M Tris, 0.192 M glycine and 0.1% SDS) at constant voltage (160 V for 2 gels).

After gel electrophoresis, gels were fixed using 12% trichloracetic acid (TCA) to increase the staining sensitivity by removing of SDS. Gels were washed in water and the protein visualization was carried out using Bio-Safe Coomassie stain. Gels were stained overnight with gentle agitation. After discarding of the staining solution, the stained background was washed with water.

4.4.1.2. 2DGE – IEF connected with SDS-PAGE

2D gel electrophoresis was performed using ReadyPrep 2D Starter Kit (Bio-Rad), ReadyStrip IPG strips 7 cm, pH 3 – 10 nonlinear (Bio-Rad) and 4 – 20 % Mini-Protean TGX gel (Bio-Rad). Aqueous extract of barley grain (variety Bojos) was dialyzed against distilled water in 3.5 kDa molecular weight cut-off Membra-Cel dialysis tubing (Serva, Heidelberg, Germany) and lyophilized. Purified grain extract (1.2 mg) was dissolved in 500 μL of rehydration/sample buffer containing 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% (w/v)

Bio-Lyte 3/10 ampholytes, Bromphenol Blue (trace). IPG strip was rehydrated (for sample loading) overnight using 125 μL of reconstituted sample. Then, IPG strip was placed in the focusing tray and IEF was running according the program described in the Table 6. The maximum current of 50 μA/strip and cell temperature of 20 °C was set.

Table 6: Running program of the IEF separation

	voltage [V]	**time [hrs:min]**	**ramp**
step 1	50	2:30	linear
step 2	100	1:00	linear
step 3	150	1:00	linear
step 4	600	0:50	linear
step 5	1000	0:45	linear
step 6	1500	0:40	linear
step 7	4000	0:50	linear
step 8	4000	until stopped	rapid

After IEF, IPG strips were equilibrated using equilibration buffers containing 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), and 20% glycerol. First, the equilibration buffer I with 2% (w/v) DTT was used for proteins reduction, and then the equilibration buffer II with 2.5% (w/v) IA was used for thiol groups of cysteines alkylation. IPG strip was washed in the running buffer for SDS-PAGE and using the agarose solution inserted on the Mini-Protean TGX gel. Gel elecrophoresis was performed at constant voltage of 160 V.

Separated proteins on the gel were stained by SYPRO Ruby protein gel stain according to the manual. After electrophoresis, gel was fixed twice for 30 minutes in the fix solution (50% methanol, 7% acetic acid). After three 10 minutes water washing steps, gel was agitated on a orbital shaker overnight in SYPRO ruby gel stain (Molecular Probes, Eugene, OR, USA). Stained gel was washed for 30 minutes in the wash solution (10% methanol, 7% acetic acid). Proteins were visualized by UV light and excised using Bio-Rad ExQuest spot cutter.

4.4.2. HPLC separations of proteins

HPLC proteins separations were performed on 1100 Series chromatograph equipped with diode array detector (Hewlett-Packard, Palo Alto, CA, USA). For all chromatographic separations, lyophilized barley extracts were dissolved in the initial mobile phase in the concentration of 50 mg/mL, centrifuged at 5.000 g for 2 min and the supernatant was filtered through a 0.45 μm PVDF Millex syringe filter units (Millipore, Billerica, MA, USA). The injection loop of 50 μL was used. Proteins were detected using UV light at 214 nm. Acquired data were processed using ChemStation software (Hewlett-Packard).

4.4.2.1. C18 RP HPLC

Reversed phase C18 liquid chromatography separation was carried on C18 column Poroshell 300SB (2.1 x 7.5 mm, 5 μm; Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed using the linear gradient of 10 – 80% acetonitrile (ACN) in 0.1% TFA for 4 min. The flow rate was set up to 1 mL/min and the column temperature to 70°C. Individual obtained fractions were collected, concentrated in Speed-Vac and lyophilized.

4.4.2.2. SEC HPLC

Barley proteins were separated on size exclusion column BioSEC-3 (7.8 x 150 mm, 3 μm; Agilent Technologies) isocratically using 150 mM phosphate buffer (pH 7.0) as the mobile phase. The flow rate was set up to 0.5 mL/min and column temperature to 25 °C. Individual obtained fractions were collected, concentrated in Speed-Vac and lyophilized.

4.4.2.3. HPLC affinity chromatography on ConA monolithic column

The enrichment of barley glycoproteins was performed on ProSwift monolithic ConA-1S affinity column, 5 x 50 mm (Dionex, part of Thermo Fisher Scientific). First, glycosylated proteins were bound to lectin stationary phase in eluent A consisting of 50 mM sodium acetate, 0.2 M sodium chloride, 1 mM calcium chloride and 1 mM of magnesium chloride (pH 7.0). After 10.5 minute, bound glycoproteins were released using eluent B pH 7.0 containing 125 mM methyl-α-D-mannopyranoside (α-MMP) in eluent A. The flow rate was set up to 0.5 mL/min and column temperature to 25 °C. Separation was repeated several times and collected proteins of unbound and bound fractions were dialyzed against distilled water using Slide-A-Lyzer Dialysis Cassettes (Thermo Fisher Scientific, Waltham, MA, USA) with 3.5 kDa cut-off, concentrated in Speed-Vac and finally lyophilized.

4.4.3. ConA affinity chromatography

Affinity chromatography was performed on manually prepared columns: a plastic column (maximal volume of 5 ml, Mo-BiTec, Goettingen, Germany) packed with 2ml of ConA bound to agarose. Each column was used only for one type of sample (grain or malt). The loading buffer (used also for equilibration and washing) was composed of 0.1 mol/L Tris (pH 7.8), 0.5 mol/L NaCl, 1 mmol/L $MnCl_2$ and 1 mmol/L $CaCl_2$. The elution buffer differed only by the addition of D-glucose in the concentration of 0.5 mol/L.

Aqueous extract from 1 g of barley grain and malt (variety Jersey) was filtered using a 0.45 μm PVDF microfilter and lyophilized. Proteins were resuspended in 2 mL of loading buffer, sonicated and filtered again. After column equilibration (with 10 mL of loading buffer), 1 mL of sample was loaded on the column and washed with 10 mL of loading buffer. Subsequently, the second half of the sample was loaded and the column was washed with 20 mL of loading buffer. The unbound protein fraction was collected. Then, bound proteins were washed of the column using 10 mL of elution buffer and collected. Both bound and unbound protein fractions were dialyzed against deionized water in 14 kDa molecular weight cut-off dialysis tubing (P-Lab, Prague, CZ), concentrated in SpeedVac and lyophilized.

4.4.4. HPLC separation of glycans

HPLC glycans separations were performed on 1100 Series chromatograph (Hewlett-Packard) coupled with an ion-trap mass spectrometry detector Esquire LC equipped with electrospray ion source (Bruker Daltonics, Bremen, Germany). Prevail Carbohydrate ES column (150 x 2 mm, 5 μm) obtained from Grace Davison Discovery Sciences (IL, USA) was used for glycan separation. The samples were dissolved in 25 μL of 50% ACN and 1 μL or 3 μL of sample was loaded on the column. The flow rate was set up to 0.2 mL/min and the separation was performed using following gradient: 70% to 50% ACN in 5 min, followed by 50% ACN for 10 min. The detection of separated glycans by ESI MS is described in the chapter 4.11.

4.5. Protein enzymatic digestion

Proteins were digested using two types of enzymes, trypsin or chymotrypsin, depending on the aim of analysis.

4.5.1. Digestion in-gel

Bands selected from 1D or 2D polyacrylamide gel were excised using scalpel or Bio-Rad ExQuest spot cutter. In-gel enzymatic digestion was performed according to Jensen et al. protocol.[88]

First, the washing of dye and other contaminants from individual gel bands was performed. The small gel pieces were washed two times by 50% ACN for 15 minutes. Then, gel pieces were shrinked by 100% ACN and subsequently rehydrated by 0,1 M ammonium bicarbonate. After 5 minutes, the equal volume of ACN was added. The solution was removed after 15 minutes and gel particles were dried down in SpeedVac.

Afterwards, gel particles were rehydrated in the reducing reagent (10 mM DTT/0.1 M ammonium bicarbonate) and incubated for 45 minutes at 56 °C. Then, the liquid was removed and replaced by the same volume of cysteine-blocking reagent (55 mM IA in 0.1 M ammonium bicarbonate). Samples were incubated for 30 minutes at room temperature in the dark. Iodoacetamide solution was removed and gel particles were washed with 0.1 M ammonium bicarbonate, and after 5 minutes, the equal volume of ACN was added. All liquid was removed after 15 minutes of incubation. If a large amount of protein was analyzed and gel particles still contained some residual staining, an additional ammonium bicarbonate/ACN washing cycle was performed.

Gel particles, completely dried down in SpeedVac, were rehydrated in the enzyme solution (25 μg of lyophilized enzyme dissolved in 2 mL of 50mM ammonium bicarbonate, 5 mM calcium chloride) at 4 °C. After 30 minutes, the remaining supernatant was removed and replaced with 20 μL of the same buffer, but without enzyme, to keep the gel pieces wet during enzymatic cleavage. The digestion was performed overnight at 37 °C.

The supernatant was removed and collected together with subsequent extracts. Peptides were extracted three times by 50% ACN in 0.1% TFA for 15 minutes in the ultrasonic bath. Extracted peptides were dried down in SpeedVac.

4.5.2. Digestion in-solution

Lyophilized proteins were re-dissolved in 50 mM ammonium bicarbonate (if necessary, 8 M urea was added). The disulphide bonds were reduced by reducing reagent (10 mM DTT in 50 mM ammonium bicarbonate; 1 μL for 10 μL of digestion solution) by incubating the mixture for 30 min at 35 °C. Then, thiol groups were alkylated by iodoacetamide (55 mM IA in 50 mM ammonium bicarbonate; 1 μL for 10 μL of digestion solution) for 20 min at room temperature in the dark. The enzymatic degradation was carried out first with LysC enzyme at 37 °C for 3 hours. Afterwards, digestion was continued overnight at 37°C after adding of trypsin. Both enzymes were added in the enzyme-to-protein ratio of 1:50 (w:w). Enzymatic digestion was stopped by addition of 5% TFA and samples were dried down in SpeedVac.

4.6. Sample purification before mass spectrometry

Prior to MALDI-TOF mass spectrometry analysis, the desalting, purification and concentration of peptides was performed using ZipTip C18 10 μL pipette tips (Millipore). This purification consists of four main steps: hydration of the C18 resin, sample loading, washing of contaminants and sample elution. First, the resin was hydrated three times with 50% ACN in 0.1% TFA (aspirated 10 μL of solution, discarded to waste, and repeated) and three times with 0.1% TFA. Then, sample dissolved in 10 μL of 0.1% TFA was slowly aspirated and expelled back into the tube for approximately ten times. Contaminants were washed by 0.1% TFA two times (aspirated 10 μL of solution, discarded to waste, and repeated). Peptides were eluted by 7 μL of 50% ACN in 0.1% TFA (five times aspirated and expelled into the same tube).

For protein purification, protein extracts were dissolved in water and centrifuged at 10,000 rpm for 20 minutes in Nanosep centrifugal devices with 3kDa cut off (Pall Life Sciences Corporation, Michigan, USA).

4.7. Isotopic labeling iTRAQ

For the iTRAQ labeling, protein digestion protocol was slightly modified according to the iTRAQ 3-Assay Duplex Trial Kit protocol. For in-solution digestion, proteins were dissolved in 20 μL of 500 mM triethylammonium bicarbonate (TEAB, pH 8.5). For protein reduction, 2 μL of 50 mM tris-(2-carboxyethyl) phosphine (TCEP) was added and samples were incubated for 1 hour at 60 °C. Then, 2 μL of 200 mM S-methyl methanethiosulfonate (MMTS) were added for protein alkylation and incubated for 10 minutes at room temperature. After enzyme digestion, the resulting peptides were incubated with iTRAQ (*m/z* 114, 117) reagents at room temperature for 90 min. After labelling, the contents of both paired samples (samples that should to be compared) were mixed together 1:1 ratio and dried completely.

4.8. Glycopeptides enrichment

Glycopeptides enrichment on ConA lectin TopTips (Glygen Corporation, MD, USA) was performed according to the manufacturer manual. Peptides obtained after in-solution digestion (without any pre-purification step) were dissolved in 20 μL of loading buffer (100 mM sodium phosphate, pH 7.0, 0,2 M NaCl). ConA TopTip was washed using the loading buffer. Sample was loaded on the tip, slowly pushed through the ConA resin and expelled back in the eppendorf tube. This procedure was repeated five times. Than, the tip was washed four times by 20 μL of washing buffer (50 mM sodium phosphate, pH 7.0, 0,2 M NaCl). Bound glycopeptides were eluted five times by 20 μL of elution buffer (0,3 M glucose in the washing buffer).

4.8.1. Purification of glycopeptides

Glycopeptides obtained from ConA TopTip enrichment were purified using carbon Supel-Tips. Carbon tips were first activated using 50% ACN and then washed by water. Sample was slowly aspirated and expelled back approximately ten times (similarly to the ZipTip C18 purification). After washing by water, bound peptides were eluted using 7 μL of 30% ACN and directly spotted on MALDI target with DHB matrix.

4.9. Deglycosylation of *N*-glycoproteins

In-gel glycoprotein deglycosylation was performed according to the enzyme product information with reduced and alkylated protein sample in Coomassie stained polyacrylamide gels. Gel pieces cutting and washing as well as the reduction and alkylation of proteins were performed as in protein in-gel protease digestion (chapter 4.5.1). PNGase F solution was added to the dried gel pieces and after 30 minutes 20 μL of water was added to cover the gel pieces. The deglycosylation was performed overnight at 37 °C. Glycan extraction was performed three times with 200 μL of water in a sonic bath for 30 minutes and all extracts were combined and dried in SpeedVac.

In-solution deglycosylation was performed according to Laštovičková et al.[81] Proteins were reduced by 50 mM DTT in 20 mM ammonium bicarbonate at 56 °C for 45 min. The deglycosylation was performed overnight at 37 °C and obtained samples were dried down in SpeedVac.

4.9.1. Purification of glycans

Samples were dissolved in 50 μL 0.1% TFA and deglycosylated proteins or peptides were captured on C18 pipette tips (maximum volume 100 μL). Glycans remaining in the unbound fraction were directly purified on activated carbon Supel-Tip, washed by 0.1% TFA and eluted by 30 % ACN in 0.1% TFA.

4.10. MALDI-TOF/TOF MS analysis

4.10.1. Sample spotting on the MALDI target

Samples were mixed with matrix solution directly on the MALDI target in the ratio 1:1. About 450 nL of matrix solution was added on the top of 450 nL of sample. Different matrices were used for different samples. The proven matrices and their mixtures are listed in the Table 7. Moreover, some additional matrices were used for optimization of glycoproteins and glycans identification and will be mentioned in appropriate chapters.

Table 7: Summary of used matrix solutions, their composition and application

matrix		concentration	solvent	application
CHCA	α-cyano-4-hydroxycinnamic acid	10 mg/mL	50% ACN in 0.1% TFA	peptides
DHAP	2,6-dihydroxyacetophenone	50 mg/mL	50% ACN in 0.1% TFA	proteins
DHB	2,5-dihydoxybenzoic acid	25 mg/mL	40% ACN in 0.1% TFA	glycopeptides, glycans
FA	ferulic acid	10 mg/mL	70% ACN in 0.1% TFA	glycopeptides
SA	sinapinic acid	20 mg/mL	70% ACN in 0.1% TFA	(glyco)proteins
DHB/SA	mixture 1:1 (v/v)			(glyco)proteins, glycans

4.10.2. MALDI-TOF/TOF MS measurement

MALDI-TOF MS experiments were performed on Applied Biosystems 4700 Proteomics Analyzer equipped with a 200 Hz Nd:YAG laser (operating at 355 nm), or on AB SCIEX TOF/TOF 5800 System equipped with a 1 kHz Nd:YAG laser (both from AB SCIEX, Framingham, MA, USA). Acquired mass spectra were processed using 4000 Series Explorer software (AB SCIEX, version 3.6, or later TOF/TOF Series Explorer version 4.1.0). Linear positive mode (for proteins analysis only) or positive reflectron mode were utilized. Accelerating voltages for MS and MS/MS measurements were set at 20 kV and 8 kV, respectively. MS/MS fragmentation was performed either with manually chosen signals, or with the highest signals in the spectrum computed by the software. MS/MS experiments were carried out by low-energy CID (collision energy set at 1 keV) where air was used as a collision gas. Mass spectra were obtained by accumulation of 2000 laser shots in MS mode and by 4000 laser shots in the case of MS/MS mode (automatically stopped after final spectrum reached high quality). The precursor ion removal was applied in MS/MS analyses.

4.10.3. Protein identification

Required mass peak lists were created by Peaks to Mascot tool in 4000 (TOF/TOF) Series Explorer software. Acquired data were submitted to the Mascot database searching.[89] Some analyses were evaluated using GPS explorer software (version 3.6) that allows faster identification of numerous samples. For protein identification, NCBInr database was used and the taxonomy restriction was set to "other green plants". Other parameters were set as follows:

- allowed missed cleavages: 1 – 3;
- fixed modification: carbamidomethyl (C);
- variable modification: oxidation of methionines;
- maximum tolerance for peptide masses: 0.5 Da;
- maximum tolerance for MS/MS fragment masses: from 0.2 to 0.8 Da;
- peptide charge: +1, monoisotopic masses;
- instrument – MALDI-TOF/TOF.

In the case of iTRAQ labelled peptides analysis, additional settings were used:

- fixed modifications: iTRAQ (N-term), iTRAQ (K);
- variable modification: iTRAQ (Y).

PMF strategy was usually not efficient for protein identification and provided only an initial view of the protein composition. Therefore, all identified proteins in this thesis were obtained after database searching of MS/MS data. Identified proteins from NCBInr database were searched in ID mapping tool of UniProt server[90] to obtain the additional information from the UniProtKB database. The summary of all identified proteins including corresponding peptides and their sequence is listed in appendix (10.1 and 10.2.).

4.11. ESI MS analysis

Analysis of glycans by ESI mass spectrometry was performed directly after glycan separation on Prevail Carbohydrate ES column (chapter 4.4.4). ESI MS and MS/MS experiments were performed with an Esquire LC ion-trap mass spectrometer (Bruker Daltonics) equipped with an ESI source. Sample solutions were introduced into the ion source via a metal capillary held at high voltage (±3.5 kV). The other instrument conditions were as follows: drying gas temperature of 350 °C; drying gas flow of 9 L/min; nebulizer pressure of 40 psi. Nitrogen was used as both nebulizing gas and drying gas. The nozzle-skimmer potential and octopole potential were modified and optimized before each experiment. Ions were scanned in the range of *m*/*z* 150 – 2200 at a scan speed of 13,000 Da/s, and 8 scans were averaged for each spectrum. The maximum number of ions allowed in the ion-trap was set at 20,000 with a maximum acquisition time of 50 ms. For MS/MS experiments the precursor ion isolation width was set to 5 Da, and the fragmentation amplitudes were varied between 0.9 and 1.55 V to reduce the intensity of the precursor ion to 10% of its initial intensity. The fragmentation time was 40 ms, the low-mass cut-off was set at default 1/3 of the precursor *m*/*z* value.

5. RESULTS AND DISCUSSION

5.1. Barley water-soluble proteins and their changes during individual stages of the malting and brewing process

The analysis of barley grain composition is important for the brewing industry, human and animal nutrition, plant breeding or cultivar identification. Protein composition of malt and intermediate products of brewing has a great technological importance, for example presence of some enzymes can influence the whole malting process. Therefore as the first aim of the doctoral thesis, the protein composition of barley grain and the changes during the malting and brewing process were investigated.

In this chapter, the attention was focused on water-soluble proteins that could pass during malt boiling into the sweet wort. Some of them survive the proteolytic enzymes activity and other undesirable conditions upon the beer production and are present in the final product, where they can influence several important beer quality properties.

Since investigated barley extracts represents a complex mixture of different water-soluble compounds as well as different kinds of proteins, it was necessary to separate this complex protein mixture before the protein MS identification. Various separation techniques were used (1D and 2D GE, C18 and SEC HPLC). Obtained results from individual protein identification and analysis of protein changes during malting and brewing are described in this chapter. Moreover, the changes of low-molecular weight barley proteins were studied using the linear mode of MALDI-TOF MS.

5.1.1. Analysis of barley grain and malt proteins by 1D gel electrophoresis

Water-extracted proteins from barley grain and malt were separated on Tris-HCl linear gradient polyacrylamide gel 4 – 20 % (Bio-Rad), and subsequently, individual protein bands were in-gel digested with trypsin and analyzed by MALDI-TOF/TOF mass spectrometry. The obtained gel with marked bands used for MS identification is shown in Figure 9 and proteins identified in individual bands are shown in the Table 8.

Barley grain is germinating during the first and essential phase of the beer production, i.e. malting. Changes in protein profile between barley grain and malt indicated the increase of the protein content and formation of new proteins. Some enzymes, for example α-amylase, β-D-xylosidase, 26 kDa endochitinase 1 or chitinase, were identified in the barley malt sample and were not detected in the grain sample. Therefore, either very small not detectable amount is present in the barley grain, or these proteins do not occur in non-germinated seed and are starting to create during malting. Focused on amylolytic enzymes, β-amylase was detected in both barley grain and malt, whereas α-amylase was identified only in the malt sample, because this enzyme is forming during the malting process.[31] Moreover, more intensive band of malt β-amylase in comparison to grain indicate increasing amount of this protein after grain germination.

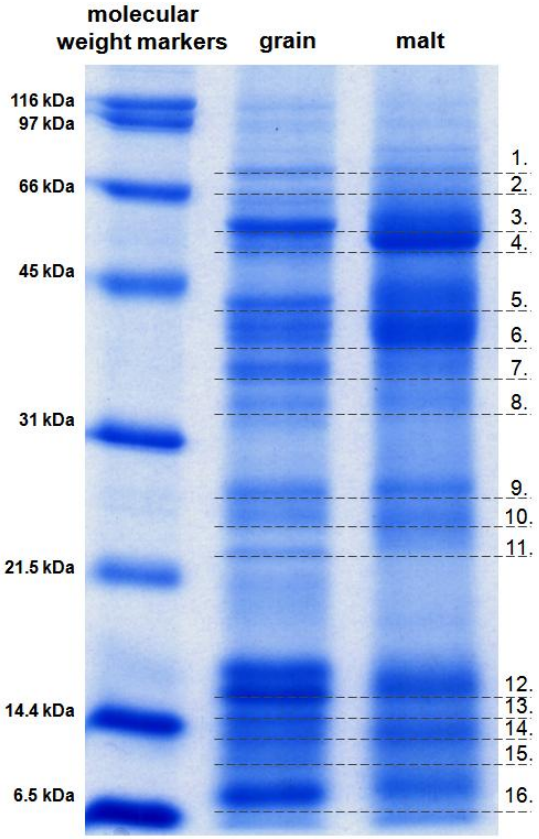


Figure 9: SDS-PAGE separation of barley grain and malt proteins. Protein bands above the marked lines were analyzed.[91]

Table 8: Summary of proteins identified by MALDI-TOF/TOF MS in barley grain and malt after SDS-PAGE and in-gel tryptic digestion.[91]
** proteins identified after database update*

spot No.	grain	malt	NCBInr entry	UniProtKB entry
1	predicted protein *		gi|326497219	F2E4C2
2		beta-D-xylosidase	gi|18025342	Q8W011
3	beta-glucosidase		gi|804656	Q40025
	beta-glucosidase [Sofia]		gi|544867	not mapped
	beta-glucosidase		gi|804656	Q40025
4	beta-amylase		gi|10953877	Q9FUK6
	beta-amylase		gi|11322499	Q9FSI3
	endosperm-specific beta-amylase 1		gi|29134855	Q84T20
	predicted protein *		gi|326493636	F2CR08
	predicted protein *		gi|326490934	F2D4W3
5		chain A, Amy2BASI PROTEIN-protein complex	gi|4699831	P04063
		alpha-amylase type B isozyme	gi|2851583	P04063
		alpha-amylase	gi|229610885	C3W8N0
		alpha-amylase	gi|166985	Q03651
		alpha-amylase	gi|229610883	C3W8M9
		alpha-amylase 1	gi|166979	Q40016
6	protein z-type serpin		gi|1310677	P06293
	protein Z (180 AA)		gi|19079	P06293
	glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic		gi|120680	P26517
	glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic *		gi|120668	P08477
	fructose-bisphosphate aldolase		gi|226316443	C1J960

spot No.	grain	malt	NCBInr entry	UniProtKB entry
7	peroxidase BP 1		gi|167081	Q40069
	chain A, crystal structure of barley grain peroxidase 1		gi|157830301	Q40069
	aldose reductase		gi|113595	P23901
		malate dehydrogenase *	gi|326490940	F2D4W6
		predicted protein *	gi|326493416	F2CQP8
8	glucose and ribitol dehydrogenase homolog		gi|7431022	F2CSK4
		1,3-beta-glucan endohydrolase GII	gi|809429	P15737
		26 kDa endochitinase 1	gi|2506281	P11955
		predicted protein	gi|326522492	F2EK36
9	26 kDa endochitinase 2		gi|116316	P23951
	chain A, the refined crystal structure of an endochitinase		gi|157834680	P23951
	triosephosphate isomerase, cytosolic		gi|2507469	P34937
10	basic pathogenesis-related protein PR5		gi|2344818	O23997
	barperm1		gi|2454602	O22462
	thaumatin-like protein TLP6		gi|14164979	Q946Z0
	thaumatin-like protein TLP7		gi|14164981	Q946Y9
		thaumatin-like protein TLP8	gi|14164983	Q946Y8
		chitinase	gi|563489	Q43765
		chitinase II	gi|9501334	Q9LEH7
		chitinase	gi|215512228	D2CVR3
11	bifunctional alpha-amylase/subtilisin		gi|18916	F2E8J4
	alpha-amylase/subtilisin inhibitor		gi|123974	not mapped
	amylase subtilisin inhibitor alpha		gi|225172	not mapped
	chain C, Amy2BASI PROTEIN-protein complex from barley seed		gi|4699833	P07596
12	alpha-amylase/trypsin inhibitor CMd		gi|585291	P11643
	CMd preprotein		gi|758343	P11643
	CMd3 protein		gi|2264392	O24000
	CMd subunit of tetrameric alpha-amylase inhibitor		gi|2266660	not mapped
	alpha-amylase/trypsin inhibitor CMa		gi|585289	P28041
	trypsin inhibitor CMe precursor		gi|1405736	P01086
	BTI-CMe1		gi|2707922	P01086
	BTI-CMe3.1 protein		gi|2707924	P01086
	trypsin inhibitor CMe		gi|85682780	P01086
	BTI-CMe2.1		gi|6634471	P01086
	alpha-amylase inhibitor BMAI-1		gi|2506771	P16968
		pathogenesis-related protein PRB1-2	gi|548588	P35792
		pathogenesis-related protein PRB1-3	gi|548589	P35792
		pathogenesis-related protein 1	gi|548592	Q05968
		PR-1a pathogenesis related protein (Hv-1a)	gi|401831	Q43489
13	alpha-amylase/trypsin inhibitor CMb		gi|585290	P32936
14	trypsin inhibitor CMc		gi|161784337	P34951
	barwin		gi|114832	P28814
	chain A, three-dimensional structure in solution of barwin		gi|159162134	P28814
15	alpha-amylase inhibitor BDAI-1		gi|123970	P13691
	trypsin/amylase inhibitor pUP38		gi|225103	not mapped
16	LTP 1		gi|19039	P07597
	chain A, non-specific lipid transfer protein 1		gi|47168353	P07597
	non-specific lipid-transfer protein 1		gi|128376	P07597
	lipid transfer protein complexed with palmitate		gi|157830246	P07597
	non-specific lipid-transfer protein *		gi|326533572	F2ED95
	predicted protein *		gi|326491097	F2EE76

In approximately 70 kDa area of grain and malt samples (spot No. 1), protein belonging to the heat shock protein 70 family was identified. This protein was identified as heat shock 70 kDa protein from maize or alternatives from rice, Arabidopsis thaliana and other plants during the experiment in 2011 (published in the journal "Kvasný průmysl")[91], but barley protein was not listed in used databases yet. In January 2013, obtained data were re-subjected to the updated NCBInr database search and the protein was identified as barley predicted protein belonging to the heat shock protein 70 family. Mass spectrum with marked peptides

corresponding to this protein is shown in Figure 10 and all peptides identified by MS/MS fragmentation are described by its sequence as well. Sequences of both maize and barley proteins were compared and align using ExPASy alignment tool SIM[92] and lalnview 3.0[93] graphical viewer. These protein sequences have 88% identity. Obtained sequence and graphical alignment is shown in Figure 11. The signal of *m/z* 1675.7 was identified as peptide A_{225}-R_{240} from maize heat shock protein. However, the corresponding signal of *m/z* 1691.7 identified as peptide S_{230}-R_{246} from barley predicted protein showed higher intensity. In addition, one peptide specific for barley protein was identified (*m/z* 1870.0, M_{336}-R_{352}).

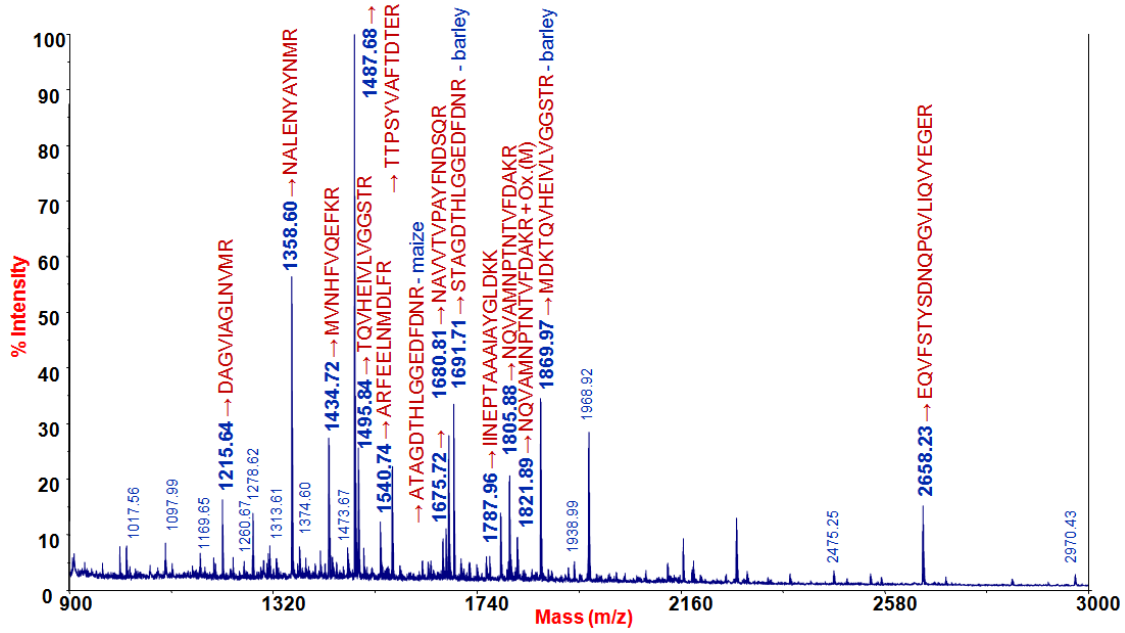


Figure 10: MALDI-TOF MS spectrum of sample No.1 corresponding to predicted protein (UniProtKB entry F2E4C2) belonging to the heat shock 70 kDa family. Identified peptides after PMF and MS/MS analysis are labelled in the spectrum.

Subsequently, data of all grain and malt samples were re-searched in the updated NCBInr database. This search led to identification of some proteins newly listed in the database (proteins labelled with a star (*) in the Table 8).

Considering these findings, several barley proteins were discovered and classified in the database during last two years. The majority of newly discovered proteins are still named "predicted proteins", and moreover, the function of some of them is still unknown. Therefore, proteomics of barley is still a current issue.

5.1.2. Analysis of barley grain proteins by 2D gel electrophoresis

To achieve the identification of higher number of barley grain proteins, the separation by 2D gel electrophoresis was performed. Proteins purified by dialysis were separated in the first dimension on the IPG strip p*I* 3 – 10, and in the second dimension on the TGX gel 4 – 20%. Obtained 2D gel is shown in figure 12. Several spots are evident in the gel, and especially low-molecular weight protein area is dominant in the whole p*I* range. Individual spots were cut-off and in-gel digested with trypsin. After ZipTip C18 purification, proteins were analyzed by MALDI-TOF/TOF MS and identified using GPS Explorer software and NCBInr database.

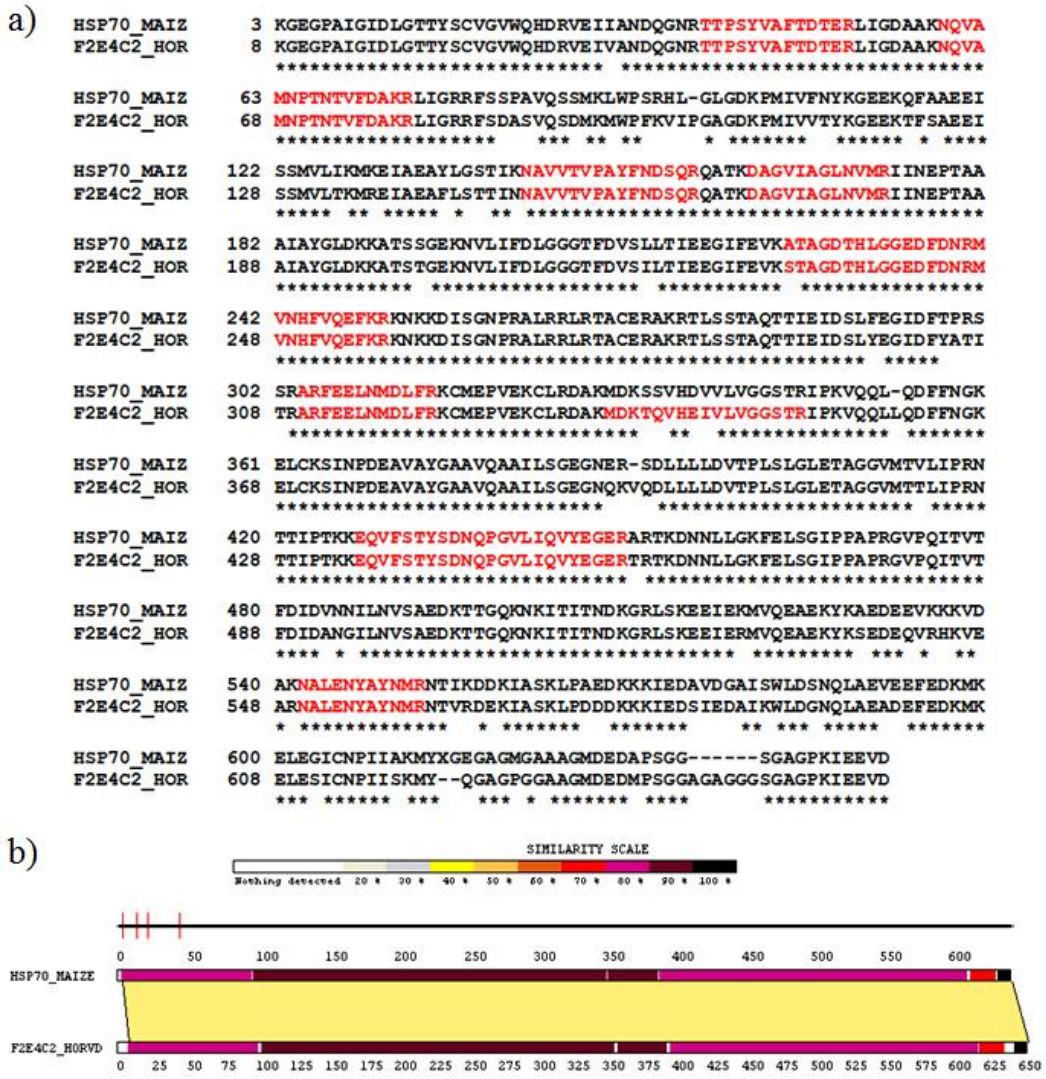


Figure 11: a) sequence alignment of maize heat shock 70kDa protein (UniProtKB entry P11143) and barley predicted protein (UniProtKB entry F2E4C2); identified peptides are highlighted in red; b) graphical alignment of maize and barley protein.

Proteins identified in individual spots are shown in the Table 9. In comparison to SDS-PAGE, the great advantage of 2D GE lies in the possibility to determine the isoelectric points of barley proteins. The theoretical p*I* of each identified protein were calculated using Expasy Compute p*I*/Mw tool[94], and are shown in the Table 9 as well. The theoretical p*I*s approximately correspond with the IEF separation for majority of identified proteins; however, there are some exceptions. For example, glyceraldehyde-3-phosphate dehydrogenase (spot No. 10), α-amylase inhibitor/endochitinase (spot No. 19) and subtilisin-chymotrypsin inhibitor CI-1A (spot No. 29) were identified in more basic area than correspond to their theoretical p*I*s. Therefore, other isoforms of the same protein or some modified proteins may be present in these spots. Moreover, these reasons may also cause the detection of some proteins in multiple spots.

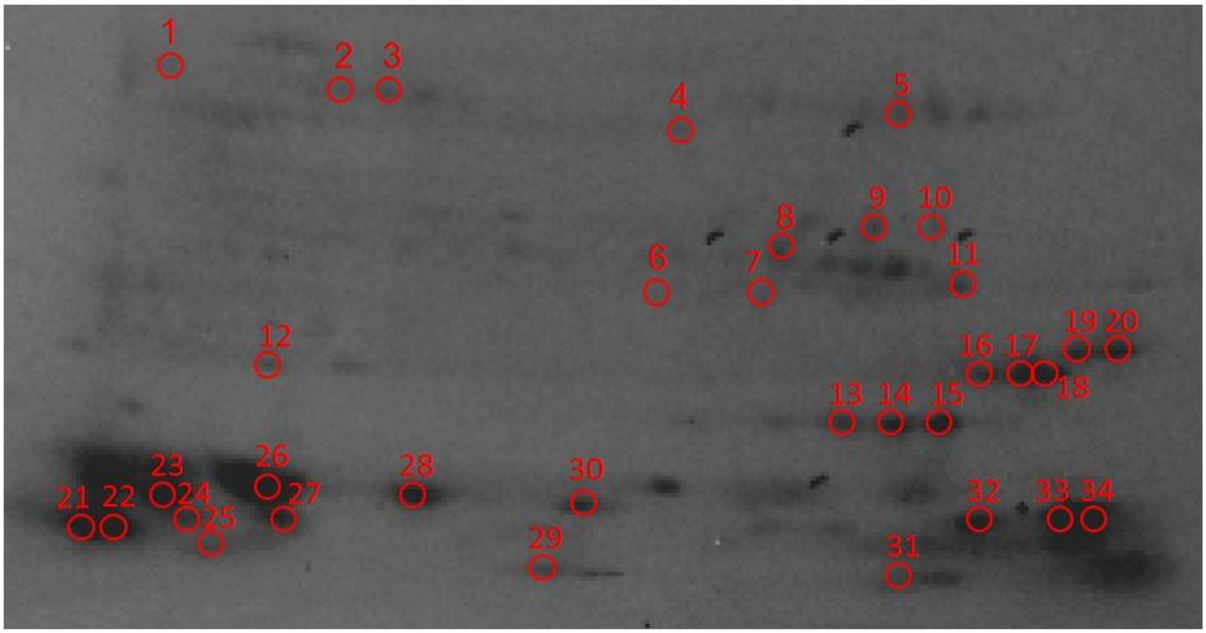


Figure 12: 2D gel of barley grain proteins. Proteins were separated in the first dimension on the IPG strip pI 3 – 10, and in the second dimension on the TGX gel 4 – 20%, and subsequently stained using SYPRO ruby stain. Marked spots indicate proteins with the most convincing identification (with the highest protein score).

Table 9: Summary of barley grain proteins identified after 2D gel electrophoresis. The theoretical pIs of each identified protein were calculated using Expasy Compute pI/Mw tool.[94]

spot No.	protein	NCBInr entry	UniProtKB entry	mass (kDa)	theoretical pI
1	tissue-ubiquitous beta-amylase 2	gi|61006818	Q4VM11	57.1	5.34
	low-molecular-weight glutenin subunit group 3 type II [Triticum aestivum]	gi|17425184	Q8W3W6	26.7	8.21
2	chain A Sevenfold Mutant Of Barley Beta-Amylase	gi|6729696	P16098	59.6	5.58
3	endosperm-specific beta-amylase 1	gi|29134857	Q84T19	59.6	5.58
	beta-amylase 1	gi|38349539	Q6SNP7	57.6	5.65
	beta-amylase	gi|10953875	Q9FUK7	59.4	5.66
4	cytosolic glutathione reductase	gi|157362219	A8CCK8	53.1	6.07
5	beta-glucosidase	gi|804656	Q40025	57.4	7.18
6	ent-kaurene synthase-like protein 2	gi|49065964	Q673F8	23.9	5.07
7	BTI-CMe2.1	gi|6634471	P01086	16.1	7.50
8	Rar1	gi|6581046	Q9SE34	25.4	7.88
9	glyceraldehyde-3-phosphate dehydrogenase cytosolic	gi|120680	P26517	36.5	6.67
10	glyceraldehyde-3-phosphate dehydrogenase	gi|126467754	A3RHT3	25.0	5.75
11	predicted protein	gi|326497617	F2EEX6	36.0	6.74
12	chitinase	gi|563489	Q43765	26.6	6.09
13	chain C Amy2BASI PROTEIN-Protein Complex	gi|4699833	P07596	22.2	7.77
14	chain C Amy2BASI PROTEIN-Protein Complex	gi|4699833	P07596	22.2	7.77
15	chain C Amy2BASI PROTEIN-Protein Complex	gi|4699833	P07596	22.2	7.77
16	basic pathogenesis-related protein PR5	gi|2344818	O23997	25.2	6.53
17	thaumatin-like protein TLP7	gi|14164981	Q946Y9	23.6	7.36
	thaumatin-like protein TLP8	gi|14164983	Q946Y8	24.3	7.83
	chain A The Refined Crystal Structure Of An Endochitinase	gi|157834680	P23951	28.2	8.83
	26 kDa endochitinase 2	gi|116316	P23951	28.5	8.83
18	thaumatin-like protein TLP8	gi|14164983	Q946Y8	24.3	7.83

spot No.	protein	NCBInr entry	UniProtKB entry	mass (kDa)	theoretical p*I*
19	alpha-amylase inhibitor/endochitinase	gi|266324	P15326	14.3	6.07
	26 kDa endochitinase 2	gi|116316	P23951	28.5	8.83
20	alpha-amylase inhibitor/endochitinase	gi|266324	P15326	14.3	6.07
	26 kDa endochitinase 2	gi|116316	P23951	28.5	8.83
21	subtilisin-chymotrypsin inhibitor CI-1A	gi|124125	P16062	8.9	5.24
	subtilisin-chymotrypsin inhibitor CI-1B	gi|124127	P16063	9.0	5.33
22	subtilisin-chymotrypsin inhibitor CI-1A	gi|124125	P16062	8.9	5.24
	subtilisin-chymotrypsin inhibitor CI-1B	gi|124127	P16063	9.0	5.33
23	trypsin/amylase inhibitor pUP13	gi|225102	not mapped	15.3	5.35
24	alpha-amylase inhibitor BDAI-1	gi|123970	P13691	16.4	5.36
25	subtilisin-chymotrypsin inhibitor CI-1A	gi|124125	P16062	8.9	5.24
	subtilisin-chymotrypsin inhibitor CI-1B	gi|124127	P16063	9.0	5.33
26	alpha-amylase inhibitor BMAI-1	gi|2506771	P16968	15.8	5.58
27	alpha-amylase inhibitor BDAI-1	gi|123970	P13691	16.4	5.36
	alpha-amylase inhibitor BMAI-1	gi|2506771	P16968	15.8	5.58
28	alpha-amylase/trypsin inhibitor CMa	gi|585289	P28041	15.5	5.86
29	subtilisin-chymotrypsin inhibitor CI-1A	gi|124125	P16062	8.9	5.24
30	predicted protein	gi|326520285	F2EJ79	15.3	6.73
31	chymotrypsin inhibitor-2	gi|158530106	A8V4D2	9.4	6.58
	subtilisin-chymotrypsin inhibitor-2A	gi|124122	P01053	9.4	6.58
32	barwin	gi|114832	P28814	13.7	7.76
33	barwin	gi|114832	P28814	13.7	7.76
	pathogenesis-related protein 4	gi|1808651	P93180	15.7	8.50
34	barwin	gi|114832	P28814	13.7	7.76

In the sample No. 1, low-molecular-weight glutenin subunit protein from wheat (*Triticum aestivum*) was identified with high protein score and three non-duplicate peptide matches after MS/MS measurement. Nevertheless, no corresponding barley protein was identified after BLAST searching[95], indicating that barley protein was not present in the NCBInr database yet. In addition, corresponding barley protein should have higher mass and lower p*I* than identified wheat protein according to the IEF separation.

Two-dimensional electrophoresis allowed more effective separation of individual proteins present in barley grain. As a result, some additional proteins were identified in comparison to one-dimensional gel electrophoresis (chapter 5.1.1, Table 8). For example, several subtilisin-chymotrypsin inhibitors were identified after 2D GE and were not detected after SDS-PAGE only. However, the protein identification after 2D GE was more difficult because of the lower protein concentration in individual samples. Also, this method was time consuming and IEF was sensitive on salt and other contaminants in the sample. Although barley grain proteins are less distributed after SDS-PAGE separation only, the MALDI-TOF MS analysis allowed the identification of multiple proteins in one spot. Therefore, 1D gel electrophoresis was used for further analyses as a more suitable method for rapid mapping of proteins occurring in the barley sample.

5.1.3. Study of protein changes during the malting process by 1D gel electrophoresis

To obtain more detailed view on protein changes during malting, samples of individual stages of malting process (grain, 1^{st} – 5^{th} day of the malting process, green malt and malt) were studied. Water-soluble proteins of individual barley samples were separated on 15% polyacrylamide gel. The analysis of proteins changes on the obtained gel (Figure 13) confirmed the previous findings, namely that the amount of some proteins is increasing and

some proteins are created in the germinated grain during the malting process. The protein lines with the most significant changes are labelled in the Figure 13. Individual proteins in grain and malt sample were identified in the chapter 5.1.1 (see Table 8). While content of β-amylase (line No. 1) seemed to increase linearly during all days of the malting process, the amount of proteins in line No. 3 (protein Z, glyceraldehyde-3-phosphate dehydrogenases, fructose-bisphosphate aldolase) probably began to increase from the fourth day of malting. The first signs of α-amylase (line No. 2) appeared in the sample from the second day of malting. Moreover, the amount of proteins in line No. 4 (26 kDa endochitinase 2, triosephosphate isomerase) seemed to be increasing, whereas the amount of α-amylase/subtilisin inhibitors (line No. 5) seemed to decrease during malting. When focussing on proteins present in the line No. 6, two strong bands were apparent in the grain sample, while only one blurry band was visible in the malt sample. In the grain sample, α-amylase/trypsin CMd inhibitors were identified in the upper band and the other identified proteins were present in the lower band (see Table 8). The fusion and blur of these two bands could be caused by formation of some new proteins (pathogenesis related proteins were detected in malt sample only), or by protein modifications created during malting.

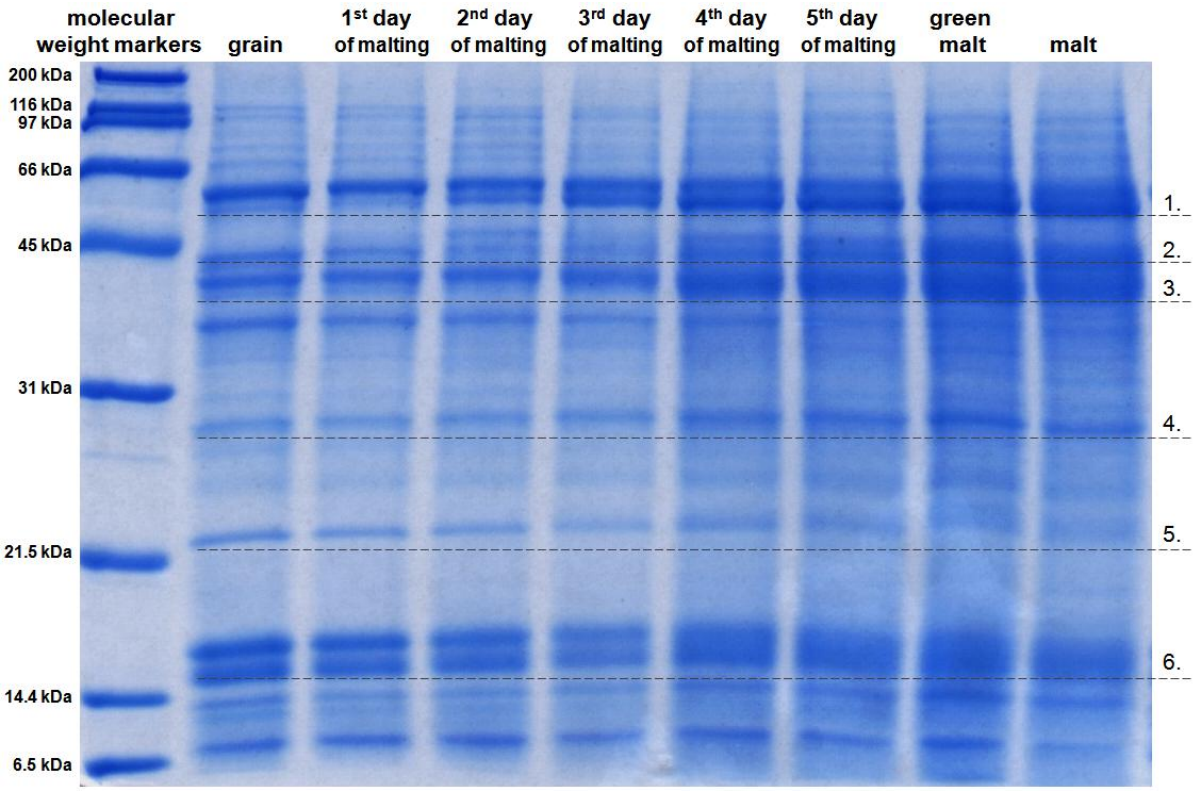


Figure 13: SDS-PAGE separation of barley proteins from individual steps of the malting process. The protein lines with the most significant changes are labelled.

5.1.4. Study of protein changes during the brewing process by 1D gel electrophoresis

Water-extracted proteins from barley grain, malt, sweet wort, wort and green beer were separated on linear gradient polyacrylamide gel 4 – 20 %, and subsequently, individual protein bands were in-gel digested with trypsin and analyzed by MALDI-TOF/TOF mass spectrometry. The obtained SDS-PAGE gel with marked bands for MS identification is shown in Figure 14 and survey of proteins identified after tryptic digestion in individual stages of the malting and brewing process is shown in the Table 10.

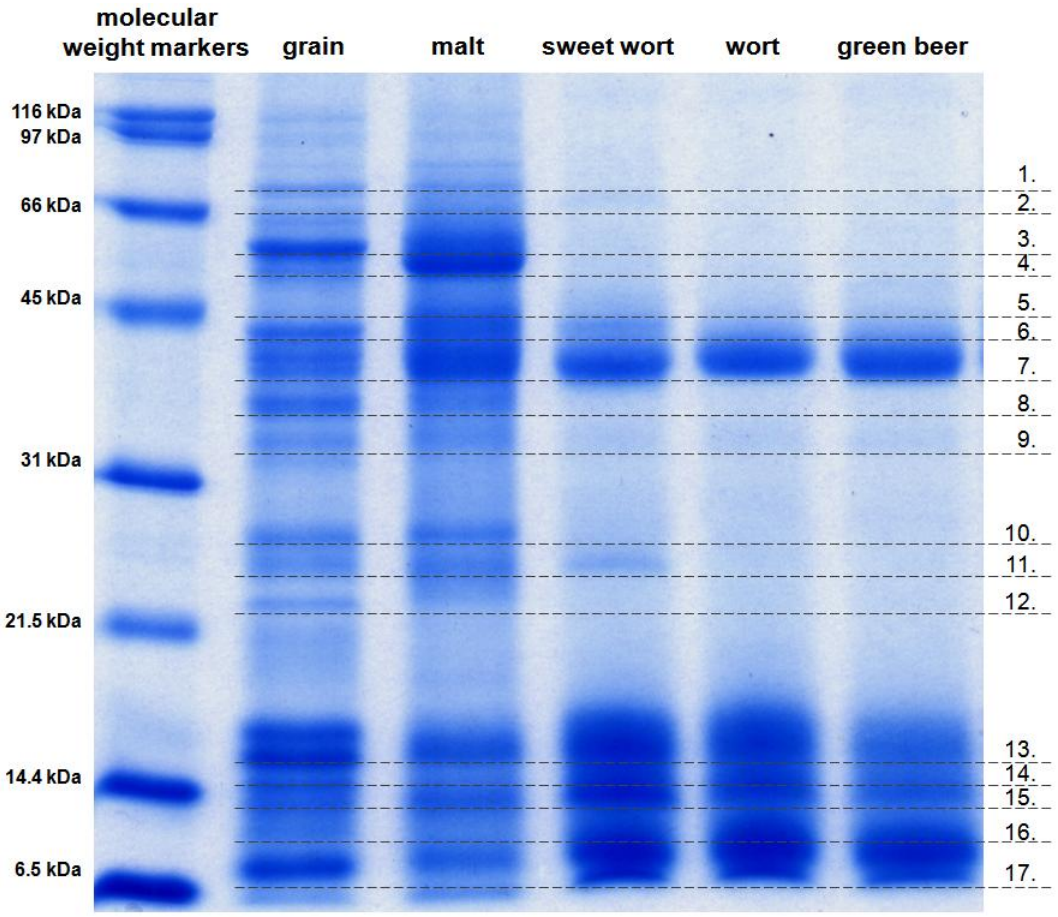


Figure 14: SDS-PAGE separation of barley proteins from individual steps of the malting and brewing process. Protein bands above the marked lines were analyzed.[91]

At the beginning of the brewing process, malt is boiled in water leading to sweet wort production. From comparison of malt and sweet wort protein profile (Figure 14) it is evident that protein amount was significantly decreasing. Many proteins were decomposed due to the high temperature and enzymatic activity of some proteases. In the sweet wort sample, α-amylase, β-D-xylosidase, barperm and thaumatin-like proteins were still identified. Considering that these proteins were not determined in the wort and green beer samples, they were apparently precipitated during brewing. In contrast to α-amylase, more temperature sensitive β-amylase[31] was not detectable in the sweet wort sample and was denatured during mashing.

Table 10: Summary of proteins identified in individual brewing stages. The presence of proteins in individual samples is highlighted by blue colour. [91]

spot No.	grain	malt	sweet wort	wort	green beer
1.	predicted protein (heat shock protein 70 family)				
2.		beta-D-xylosidase			
3.	beta-glucosidase				
4.	beta-amylase				
5.					enolase 1 [*Saccharomyces cer.*]
6.		chain A, Amy2BASI PROTEIN-Protein Complex alpha-amylase			
7.	protein z-type serpin glyceraldehyde-3-phosphate dehydrogenase, cytosolic fructose-bisphosphate aldolase				
8.	aldose reductase peroxidase BP 1				
9.	glucose and ribitol dehydrogenase homolog	26 kDa endochitinase 1			
10.	26 kDa endochitinase 2 triosephosphate isomerase, cytosolic				
11.	barperm1 thaumatin-like protein TLP6; TLP7 basic pathogenesis-related protein PR5				
12.	thaumatin-like protein TLP8 chitinase alpha-amylase/subtilisin inhibitor bifunctional alpha-amylase/subtilisin inhibitor chain C, Amy2BASI PROTEIN-Protein Complex				
13.	alpha-amylase/trypsin inhibitor CMd CMd3 protein alpha-amylase inhibitor BMAI-1 trypsin inhibitor CMe BTI-CMe2.1 alpha-amylase/trypsin inhibitor CMa				
14.	pathogenesis-related proteins alpha-amylase/trypsin inhibitor CMb				
15.	barwin trypsin inhibitor CMc				
16.	alpha-amylase inhibitor BDAI-1 trypsin/amylase inhibitor pUP38				
17.	non-specific lipid-transfer protein 1 lipid transfer protein complexed with palmitate				

In sweet wort, wort and green beer samples, two general protein areas are evident (Figure 14). In the first intensive area about the molecular weight of 40 kDa, protein Z (belonging to the group of PR-6 proteins) was identified. The second area occurs in range from 20 to 6 kDa, where several of low-molecular weight proteins were identified, namely protease/α-amylase inhibitors (PR-6) and ns-LTP (PR-14). While protein Z content seemed to be stable during the whole brewing process, the content of low-molecular weight proteins was decreasing, according to the less intense spots on the gel and less intense peptides intensity in MS spectra as well. Obtained results confirmed that identified barley proteins from PR-6 and PR-14 groups are temperature stable and protease resistant, they can get up to the final product where they could affect the quality properties. In the green beer sample, one protein originating from *Saccharomyces cerevisiae* yeast was identified as well.

These results were published in 2011 in the journal Kvasný průmysl.[91]

1D gel electrophoresis represents a suitable method for successful monitoring of changes in the protein profile during individual steps of the malting and brewing process. The representation of individual proteins in the sample may be determined visually from the intensity of corresponding protein spots. Nevertheless, the spot intensity may be influenced also by other factors (for example protein modifications). The exact quantification of individual proteins and/or their changes requires some additional equipment or method, for example the iTRAQ labelling for relative quantification described in the chapter 5.3.

5.1.5. Study of protein changes during the malting and brewing process by HPLC C18 separation

Water-extracted proteins from barley grain, malt, sweet wort, wort and green beer were separated on C18 reversed phase HPLC column. The changes in protein profile during the malting and brewing process are shown in chromatograms (Figure 15). Marked grain and malt fractions were collected, in-solution digested with trypsin, and analyzed by MALDI-TOF/TOF MS after peptide purification. Identified proteins are summarized in the Table 11. The MS identification of proteins in the first two HPLC fractions after tryptic digest resulted in very poor spectra and no proteins were identified. Therefore, intact proteins from these fractions were analyzed by MALDI-TOF MS in linear mode. In the first and second fraction, ns-LTP2 and ns-LTP1b were detected, respectively. However, this identification is not as accurate as the identification of proteins after MS/MS fragmentation of tryptic peptides. Considering the large area of these two peaks, also some additional proteins (probably low-molecular weight ones) may be present in them.

Table 11: Proteins identified in barley grain and malt after reversed phase C18 HPLC separation, tryptic in-solution digestion and MALDI-TOF/TOF MS analysis.[91]

peak No.	grain	malt	NCBInr entry	UniProtKB entry
3	alpha-amylase/trypsin inhibitor CMb		gi|585290	P32936
	trypsin/amylase inhibitor pUP38		gi|225103	not mapped
	alpha-amylase/trypsin inhibitor CMd		gi|585291	P11643
	CMd3 protein		gi|2264392	O24000
	alpha-amylase inhibitor BMAI-1		gi|2506771	P16968
4	bifunctional alpha-amylase/subtilisin inhibitor		gi|18916	F2E8J4
	chain C, Amy2BASI PROTEIN-protein complex from barley seed		gi|4699833	P07596
5	putative avenin-like a precursor		gi|326501830	F2EGD5
6	subtilisin-chymotrypsin inhibitor-2A		gi|124122	P01053
	chymotrypsin inhibitor-2		gi|158530106	A8V4D2
7	26 kDa endochitinase 2		gi|116316	P23951
	chain A, the refined crystal structure of an endochitinase		gi|157834680	P23951
8	aldose reductase		gi|113595	P23901
	chain A, crystal structure of barley grain peroxidase 1		gi|157830301	Q40069
9	fructose-bisphosphate aldolase		gi|226316443	C1J960
		alpha-amylase type B isozyme	gi|2851583	P04063
10	beta-amylase		gi|10953877	Q9FUK6
11	beta-glucosidase		gi|804656	Q40025
12	protein z-type serpin		gi|1310677	P06293

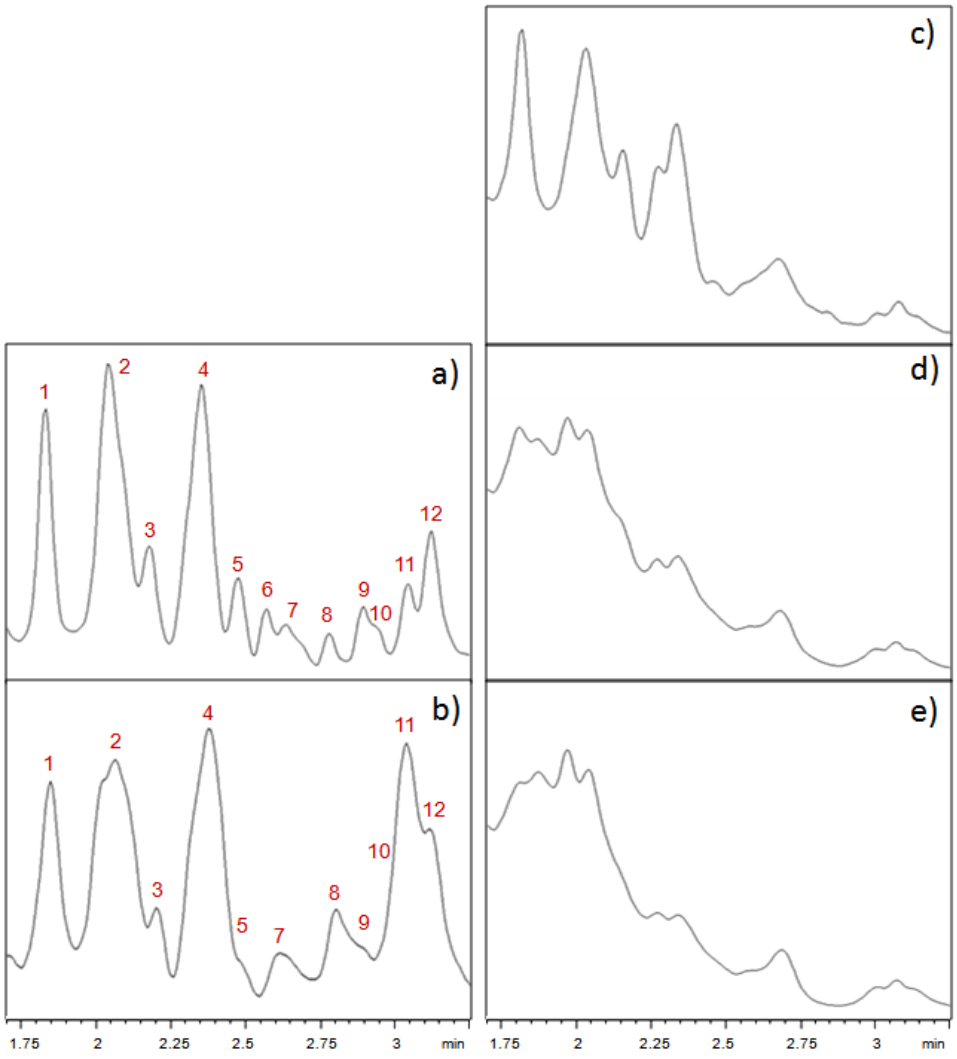


Figure 15: Chromatograms from HPLC C18 separation of barley water-soluble proteins, showing the protein changes during the malting and brewing process: a) grain; b) malt; c) sweet wort; d) wort; e) green beer. Marked fractions from grain and malt were collected and analyzed.[91]

Protein profile changes during the malting and brewing process observed on HPLC C18 chromatograms are similar to the protein profile changes on the SDS-PAGE gel (Figure 14). In the chromatogram of barley grain sample, large amount of relatively narrow peaks is evident, while in malt sample, the higher amount of proteins resulted in formation of broader peaks. In the grain and malt samples, identical proteins were identified in corresponding peaks, only chymotrypsin inhibitors and putative avenin-like a precursor were not detectable in malt (peaks No. 5 and 6), and α-amylase was not identified in grain sample (peak No. 9) because this enzyme is formed during malting. The biggest changes in the grain and malt chromatographic protein profiles were observed in the peaks No. 10 and 11, corresponding to β-glucosidase and β-amylase, respectively. In the malt sample, these two peaks were joined in one broader peak. The cause is probably the increasing amount of β-amylase during malting, which is in agreement with our previous results (chapter 5.1.1).

During mashing and mainly brewing, many proteins are denatured. In the HPLC C18 chromatogram of sweet wort sample, disappearance of some peaks was obvious. Especially the large peak No. 11 in malt sample corresponding to β-glucosidase and β-amylase was disappeared. However, in contrast to wort sample, some proteins were still evident in sweet wort and were proteolytically digested or precipitated during the wort boiling. Wort and green beer sample showed almost identical chromatogram, and according to identification after SDS-PAGE, both of these samples contain ns-LTPs, protein Z and other protease/α-amylase inhibitors.

One separation step of the reversed phase C18 HPLC separation of barley proteins took four minutes, and therefore, this method represents a suitable method for rapid barley protein separation. The separation of barley grain was the most efficient as it led to 12 relatively sharp peaks. Proteins were separated according to their hydrophobicity on the reversed phase column; however, the influence of separation according to the molecular mass was also obvious, as was described in the literature.[3] Obtained results were published in 2011 in the journal Kvasný průmysl.[91]

5.1.6. Study of protein changes during the malting process by HPLC SEC separation

Barley grain and malt aqueous extracts were separated on size exclusion BioSEC-3 column. Marked fractions (Figure 16) were collected, concentrated in SpeedVac and lyophilized. Then, individual fractions were in-solution digested with trypsin and analyzed by MALDI-TOF/TOF MS after ZipTip C18 purification. Identified proteins in individual grain and malt fractions are shown in the Table 12.

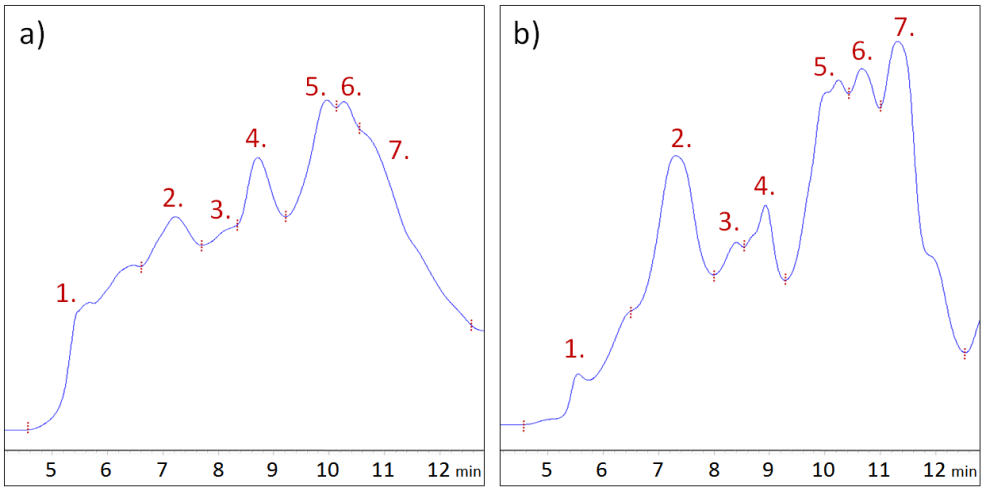


Figure 16: HPLC SEC separation of water-soluble proteins of barley a) grain; and b) malt. Marked fractions were collected and analyzed.

Several proteins were identified in barley grain and malt, including some proteins yet unidentified in our previous studies (e.g. some predicted proteins, proteins in peak No. 3, or dehydrin in peak No. 7). Barperm1, thaumatin-like proteins and pathogenesis-related protein PR5 were identified in grain sample only; however, after SDS-PAGE they were identified in malt as well. As a possible explanation, the presence of probably huge amount of α-amylase in this malt fraction could make worse the identification of these proteins.

Table 12: Summary of identified proteins in barley grain and malt after SEC HPLC separation, tryptic in-solution digestion and MALDI-TOF/TOF MS analysis.

peak No.	grain	malt	NCBInr entry	UniProtKB entry
1.	predicted protein		gi|326520537	F2EJK5
2.	beta-amylase		gi|10953877	Q9FUK6
	beta-glucosidase		gi|804656	Q40025
	protein z-type serpin		gi|1310677	P06293
3.	late embryogenesis abundant protein B19.1A		gi|547817	Q05190
	grain softness protein		gi|54661662	Q5ITH7
	predicted protein		gi|326502266	F2DJC5
	glucose and ribitol dehydrogenase homolog		gi|7431022	not mapped
	predicted protein		gi|326493416	F2CQP8
	aldose reductase		gi|113595	P23901
		predicted protein	gi|326506996	F2DKF4
4.	cold-regulated protein		gi|10799810	Q9FSI8
		alpha-amylase type B isozyme	gi|2851583	P04063
	predicted protein		gi|326520285	F2EJ79
	barperm1		gi|2454602	O22462
	thaumatin-like protein TLP7		gi|14164981	Q946Y9
	thaumatin-like protein TLP6		gi|14164979	Q946Z0
	basic pathogenesis-related protein PR5		gi|2344818	O23997
5.	alpha-amylase inhibitor BDAI-1		gi|123970	P13691
	trypsin/amylase inhibitor pUP13		gi|225102	not mapped
6.	barwin		gi|114832	P28814
	thaumatin-like protein TLP8		gi|14164983	Q946Y8
7.		dehydrin	gi|6017948	Q9ZTR8
	Amy2BASI PROTEIN-Protein Complex From Barley Seed		gi|4699833	P07596
	thioredoxin H2		gi|119390312	P07596
	alpha-amylase/subtilisin inhibitor		gi|123974	P07596
	alpha-amylase/trypsin inhibitor CMa		gi|585289	P28041
	alpha-amylase/trypsin inhibitor CMb		gi|585290	P32936
	trypsin inhibitor CMe		gi|85682780	P01086
	putative avenin-like a precursor		gi|326501830	F2EGD5

The biggest difference between grain and malt profiles was observed in peaks No. 3, where three high abundant proteins were identified: β-amylase, β-glucosidase and protein Z. This peak was significantly increasing during malting. The more detailed view on the changes of these proteins obtained by iTRAQ relative quantification method is shown in chapter 5.3.1. In addition, an significant increase was observed also in grain and malt peaks No. 7, where several α-amylase/trypsin inhibitors were identified, indicating the increasing amount of one or more of these proteins during malting.

In comparison to separation on reversed phase C18 column (chapter 5.1.5), individual protein fractions were more separated from each other after C18 HPLC than after SEC HPLC. Moreover, C18 HPLC separation was faster, which is given mainly by different column dimensions. The advantage of SEC HPLC lies in the ability to approximately estimate the molecular weight of proteins.

5.1.7. Changes in low-molecular weight protein profile during malting and mashing

For the more detailed characterization of changes of low-molecular weight barley proteins during malting and mashing, the linear mode of MALDI-TOF mass spectrometer was used. Aqueous extracts of grain, malt and sweet wort were used for investigation of low-molecular weight protein profile changes during malting and mashing. Individual samples were purified via Nanosep centrifugal devices for MS signal improvement due to desalting and removing of compounds with molecular weight lower than 3 kDa. Samples were spotted on MALDI target in ratio 1:1 with DHAP matrix solution.

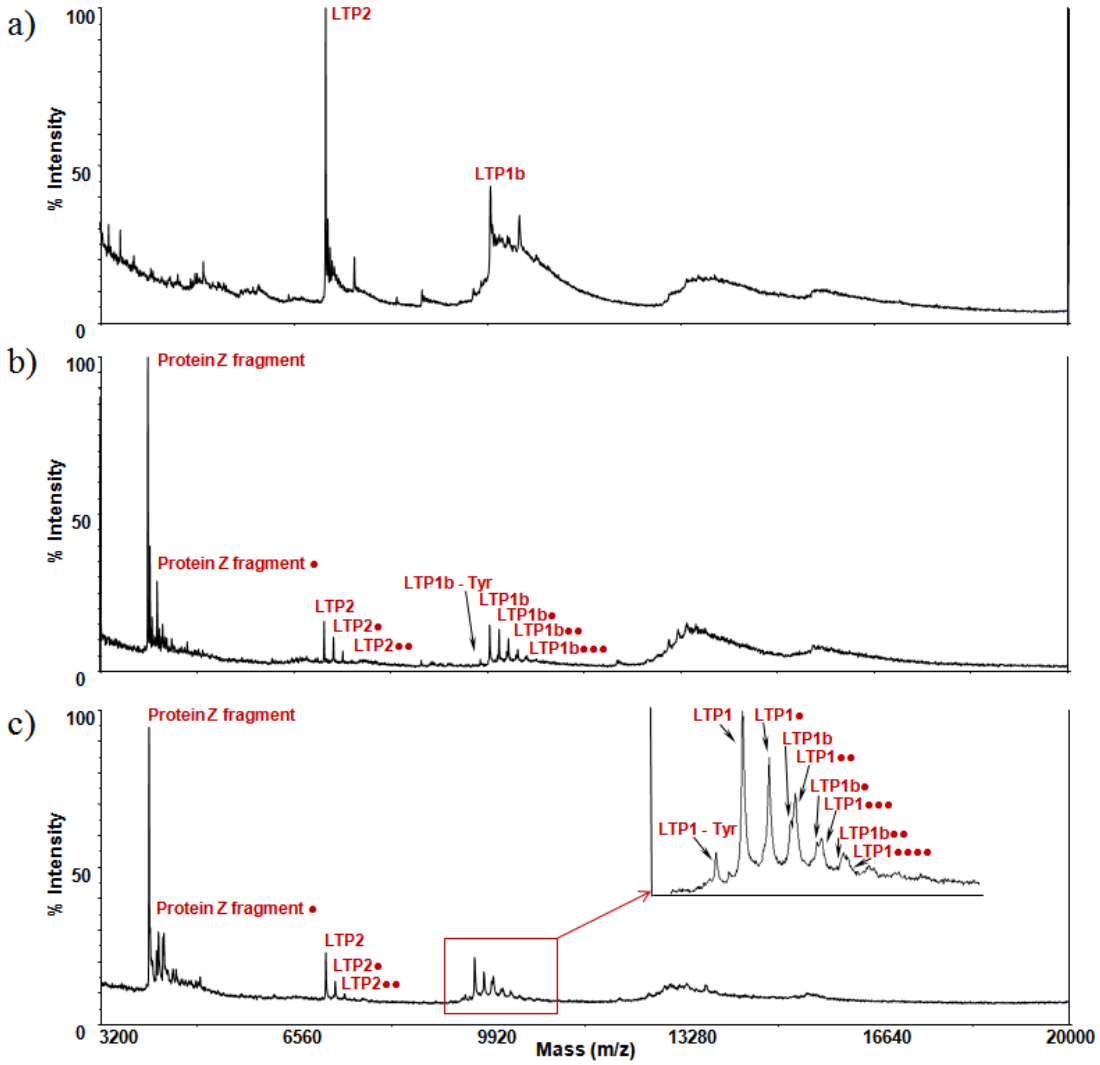


Figure 17: MALDI-TOF linear mode MS spectra of low-molecular weight proteins from a) grain; b) malt; c) sweet wort. The non-enzymatic glycation by one hexose unit is marked by a dot sign (●).[91]

Acquired MALDI-TOF spectra labelled with names of corresponding proteins are shown in Figure 17. Following proteins belong among major proteins in the low-mass 3.2 – 20 kDa area: protein Z fragment (C-terminal 363 – 399 fragment[49]; 4.03 kDa), LTP1 (9.69 kDa), LTP2 (7.10 kDa) and LTP1b (LTP1 with bound 294 Da lipid-like molecule 9-hydroxy-10-oxo-12(Z)-octadecenoic acid[57]; 9.98 kDa). LTP2 and LTP1b were detected in all three

samples. Fragment of protein Z was not detectable in the grain sample. However, it is formed during malting and resulted in the most intensive peak of the malt and sweet wort MS spectra. This proteolytic cleavage is probably caused by the interaction between protein Z and serine proteases and supports the creation of heat and protease stable molecule of protein Z that survives the brewing process and is present in beer.[49,50] The sequence of protein Z with marking of fragment cleavage is shown in Figure 28 (chapter 0).

While only lipid-modified form of LTP1 (known as LTP1b) was detected in grain and malt samples, LTP1 form was more intensive than LTP1b in the sweet wort sample. This indicates that the lipid-protein bond is probably breaking up during mashing. This conclusion is in agreement with findings obtained by Perrocheau et al.[51], who have discovered that glycation induced by Maillard reaction was more heat stable than the ester linkage of the lipid like adduct of the barley LTP1.

All discussed proteins are non-enzymatically glycated after the malting process. A hexose unit (very likely glucose) is bound to the protein, which leads to the increase of the molecular mass about 162 Da. From obtained spectra it is evident that MALDI-TOF MS operated in the linear mode provide a suitable method for study of glycation of low-molecular weight proteins. This protein modification will be further analyzed and discussed in detail in the chapter 5.5.

Low-molecular weight barley proteins and the changes they have undergone during the malting and mashing can be successfully monitored using the MALDI mass spectrometer in the TOF linear mode. Obtained results were published in 2011 in the journal Kvasný průmysl.[91]

5.2. Barley prolamins (hordeins) and their changes during malting

Barley prolamins (hordeins) are alcohol-soluble storage proteins of barley grain and the main protein fraction of barley endosperm.[62] Hordeins are present in the protein matrix that surrounds the starch granules within the cells of the endosperm. Degradation of the hordein in this matrix during malting is necessary to allow starch degrading enzymes access to the starch, which facilitates complete starch hydrolysis.[63] The aim of this study was to investigate the effect of the malting on hordein composition.

Hordeins were extracted by 60% ethanol and 2% DTT from samples of individual stages of the malting process: barley grain, 1st to 5th day of malting, green malt and malt. Obtained proteins were separated by SDS-PAGE on 12% polyacrylamide gel (Figure 18). Grain and malt proteins were in-gel digested with chymotrypsin and analyzed by MALDI-TOF/TOF MS.

Table 13: Identified proteins from barley prolamin fraction.[96]

spot No.	protein	NCBInr entry	UniProtKB entry
1.	D hordein	gi|1167498	Q40054
2.	C hordein	gi|167016	Q40037
3.		gi|442524	Q41210
4.	C hordein	gi|442524	Q41210
5.	B1 hordein	gi|82548225	Q2XQF0
6.	B3 hordein	gi|123459	P06471
7.	γ hordein	gi|123464	P17990

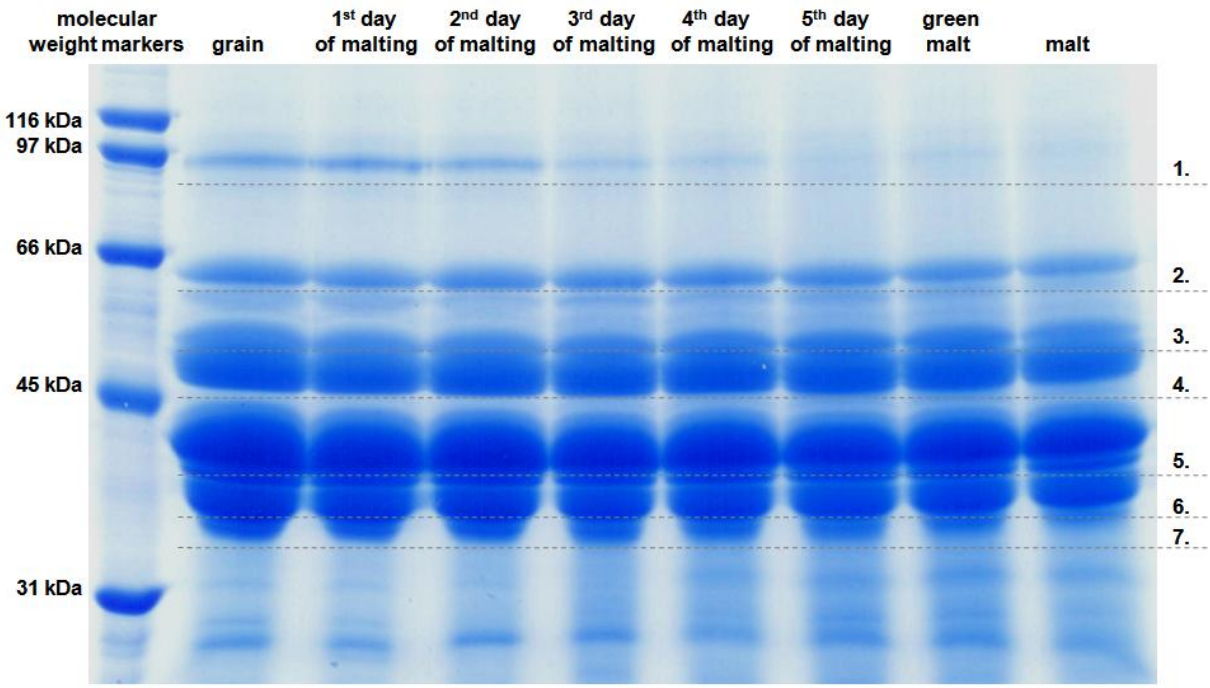


Figure 18: SDS-PAGE separation of barley prolamins (hordeins) from individual steps of the malting process. Protein bands above the marked lines were analyzed.

Although trypsin is a preferred enzyme for protein identification, hordeins contain very small number of arginine and lysine, which cause the formation of peptides with inappropriate molecular mass for subsequent MS analysis. Therefore, chymotrypsin was used for enzymatic cleavage of hordeins and was found as a suitable enzyme for this type of analysis. The MALDI-TOF/TOF MS analysis and subsequent database searching resulted in identification of several barley hordeins (Table 13). In contrast to various proteins found in the fraction of water-soluble proteins, proteins in alcohol-soluble prolamin fraction can be divided in only four general groups: D hordeins, C hordeins, B hordeins and γ hordeins. According the spot intensities on the gel, B and C hordeins are the major proteins, which is in good agreement with theory.[62]

The SDS-PAGE separation of proteins from individual stages of the malting process provided the basic information about the protein changes during malting. The slight decrease of hordeins content during malting is evident from the weakening intensity of hordeins spots. The most rapid decrease was noticed in spot number 1 corresponding to D hordein. From the 5th day of malting, D hordein is almost undetectable after Coomassie gel staining. This finding was supported also by MS analysis of corresponding peptide digests. D hordein is almost completely degraded during the malting, which can improve malting because D hordein displayed the strongest negative correlation with malt quality according to Howard et al.[63] Protein spots from line No. 4 corresponding to C hordein were subsequently used for characterization of protein changes during malting using the iTRAQ relative quantification method (chapter 5.3.2).

Obtained results were published in 2012 in the European Journal of Mass Spectrometry.[96]

5.3. Relative quantification of barley proteins using iTRAQ method

The iTRAQ labelling method was used for the relative quantification of barley protein changes during malting. Used iTRAQ method was based on two reagents of the same mass (isobaric tags) that contained different reporter groups – *m/z* 114 and *m/z* 117. The analysis was based on labelling of protein proteolytic digests by these reagents. Then, paired samples were mixed and analyzed by MALDI-TOF MS. The mass of all labelled peptides was higher by 144.1 Da, but after MS/MS fragmentation of these peptides, the fragments of the reporter groups (*m/z* 114 corresponding to the first sample and *m/z* 117 corresponding to the second sample) were present in the MS/MS spectra. By the comparison of peak areas of these fragments, the relative quantification of samples labelled by corresponding reagent was performed.

5.3.1. Relative quantification of selected barley albumins

From the HPLC SEC separation of barley grain and malt water-soluble proteins (chapter 5.1.6), the significant area increase of peak number 2 was obvious (Figure 16). Since three proteins were identified in this fraction (namely β-amylase, β-glucosidase and protein Z), iTRAQ method was used to obtain detailed view on changes of these particular proteins after the malting process.

β-Amylase belong between amylotic enzymes that are involved in hydrolysis of α1-4 glycosidic bounds in starch, glycogen and other polysaccharides, and therefore, they are responsible for the increasing amount of fermentable sugars in sweet wort.[31] β-Glucosidase is able to degrade fungal cell wall polysaccharides and therefore may provide a protection against microbial invasion of germinated barley grain.[97] Protein Z is important for the quality of beer, especially improves the foaming properties.[8] Accordingly, all these three proteins are important for high quality of malt and/or beer.

Selected fraction of grain and malt was collected and proteins were in-solution digested with trypsin, and subsequently, the iTRAQ labelling was performed. The reagent of *m/z* 114 was used for barley grain sample and the reagent of *m/z* 117 for malt sample. Then, aliquots of grain and malt sample were mixed together in the ratio 1:1 and dried down completely. Samples were purified using C18 ZipTips and analyzed by MALDI-TOF/TOF mass spectrometry.

Obtained MS/MS data were subjected to database searching, and as a result, six peptides of β-amylase, four peptides of β-glucosidase and two peptides of protein Z were identified. Identified peptides were used to relative quantification analysis. The MS/MS fragmentation spectrum of each peptide was measured three times and the data evaluation of each spectrum provided a ratio of *m/z* 114 peak area towards *m/z* 117 peak area (or ratio of grain towards malt), and the three ratios of one peptide were averaged. Obtained results are presented graphically in Figure 19.

The amount of all three proteins presented in investigated HPLC SEC fraction is increasing during the malting process. By averaging of obtained ratios of each protein, a total ratio of grain and malt protein content was obtained. If the malt protein content is expressed as 100 %, approximately 60 % of malt β-amylase is present in grain sample. Accordingly, approximately 80 % of malt β-glucosidase and 83 % of malt protein Z are present in grain. It follows that the content of β-amylase is increasing more rapidly in comparison to β-glucosidase and protein Z.

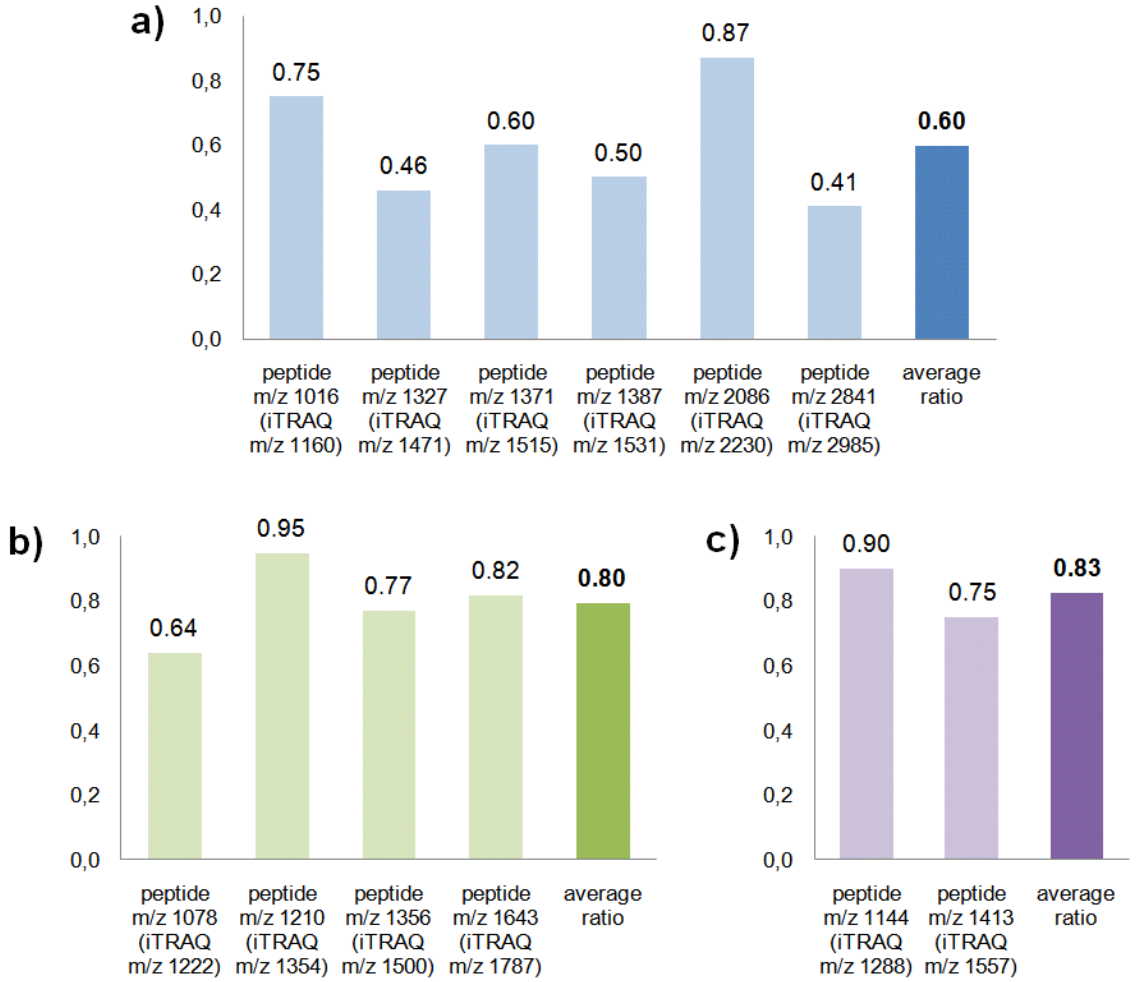


Figure 19: peak area ratios (grain towards malt) of iTRAQ modified peptides corresponding to: a) β-amylase; b) β-glucosidase; and c) protein Z.

5.3.2. Relative quantification of C hordein

The analysis of hordein profiles of barley grain, individual stages of the malting process and malt after SDS-PAGE (chapter 5.2) indicated that the content of C hordein is decreasing during the malting process (line number 4 in Figure 19). To obtain more detailed view on these changes, relative quantification using iTRAQ labelling method was performed. Bands of C hordein in line number 4 were excised, in-gel digested with chymotrypsin, and peptides obtained by extraction from the gel were subjected to the iTRAQ labelling process. Reagent of *m/z* 114 was used for samples of individual malting stages, and reagent of *m/z* 117 was used for grain sample only. After labelling, aliquots of grain sample were mixed with samples of individual malting stages in the ratio 1:1 and dried down completely. Prior to MALDI-TOF/TOF mass spectrometry, samples were purified using ZipTip C18 pipette tips.

Three iTRAQ labelled peptides belonging to C hordein (Q41210) were identified after MS/MS fragmentation and database searching. The signals at *m/z* 2133, *m/z* 2247 and *m/z* 2300, iTRAQ modified with a mass increment of 144.1 Da, correspond to C hordein unmodified peptides of 1989 Da, 2103 Da and 2156 Da, respectively. For one sample, the fragmentation MS/MS spectrum of each modified peptide was measured three times. The representation of C hordein within malting is expressed by ratios of peak areas

at *m/z* 114/117 of individual modified peptides, where *m/z* 117 represent grain and *m/z* 114 represent the following stages of the malting process. The average ratios corresponding to individual peptides were calculated for each sample and are presented graphically in Figure 20.

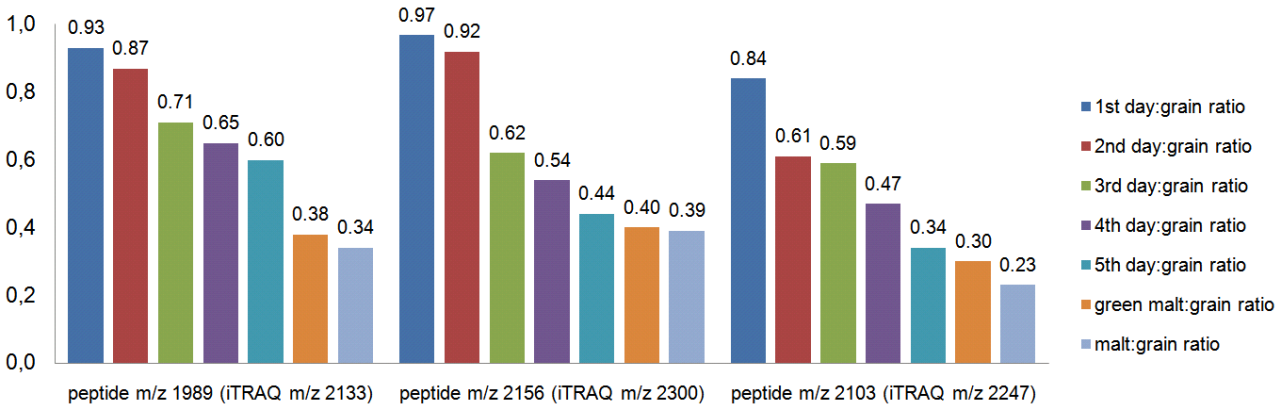


Figure 20: Peak area ratios (individual malting stages towards grain) of iTRAQ modified peptides corresponding to C hordein.[96]

Showed data confirmed that the amount of C hordein is significantly decreasing during the malting process. Moreover, when the three ratios of individual peptides were averaged, a detailed view on protein decrease was obtained. The amount of C hordein in malt represented 35 % of the initial amount in barley grain (Figure 21).

This study was published in 2012 in the European Journal of Mass Spectrometry.[96]

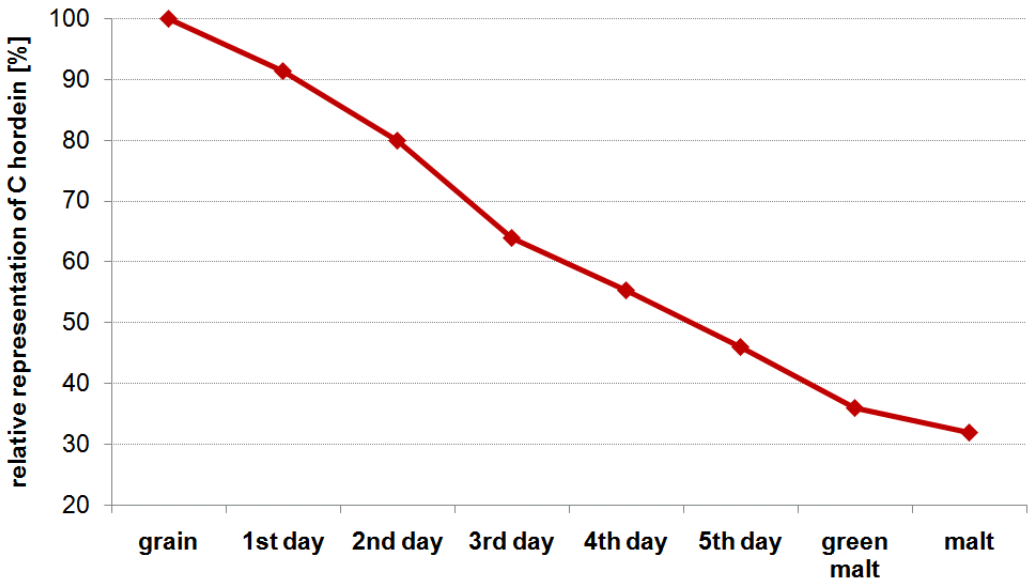


Figure 21: Relative representation of C hordein within individual stages of the malting process. The amount of C hordein is decreasing during malting up to 35 % of the initial quantity.[96]

Used iTRAQ technique represents a suitable method for relative quantification of protein changes during the malting process. It can be successfully used for HPLC separated and in-solution digested proteins (chapter 5.3.1), as well as for SDS-PAGE separated proteins after in-gel digestion (chapter 5.3.2). To compare these two approaches, iTRAQ quantification after SDS-PAGE seems to be more suitable and faster method for mapping of protein changes during the whole malting process by monitoring of the individual malting step.

5.4. Comparison of selected barley varieties

Distinction between individual barley varieties is crucial for malsters and beer producers, as well as for biochemical studies. Protein composition of barley grain affects the quality of malt and beer.[98] For the production of authentic Czech beer, only eight barley varieties are allowed (Tolar, Malz, Bojos, Blaník, Advent, Aksamit, Calgary and Radegast) according to recommendation of the RIBM.[13] Therefore, study of relationships between these approved varieties and other barley varieties is desirable. Barley variety discrimination is often performed by SDS-PAGE separation of alcohol-soluble proteins (hordeins), and by comparison of hordein profile of individual varieties. This method can be used in many laboratories because it does not require expensive equipments. Nevertheless, some European barley varieties remain undistinguishable, since they are closely related and have very similar protein pattern.[99] Moreover, Šalplachta et al.[62] published a variety discrimination method by MALDI-TOF analysis of intact hordeins. The main advantage of this approach is quite short time of analysis in contrast to conventional electrophoretic method. MS analysis of hordein extracts provides characteristic protein profile for each barley variety. However, this method requires expensive equipment.

Therefore, a simple, rapid and robust method for varieties discrimination is still needed. For this purpose, several spring barley varieties were studied to find some differences in their protein composition using one simple method.

5.4.1. Comparison of protein profiles of individual barley varieties using C18 HPLC

For this study, grain and malt samples of six European barley varieties were compared: Jersey (hulled variety with good malting properties), Tolar, Blaník, Bojos, Malz (hulled varieties allowed for the production of beer with PGI Czech beer) and AF Lucius (non-malting hulles variety). Aqueous extracts of barley grain and malt of individual varieties were separated on C18 Poroshell 300SB HPLC column in the linear gradient of ACN in 0.1% TFA. Obtained chromatograms of barley grains and malts are shown in Figures 22 and 23, respectively. The differences between chromatograms of barley grain and malt are discussed in the chapter 5.1.5 and the identification of individual grain and malt protein is shown in theTable 11.

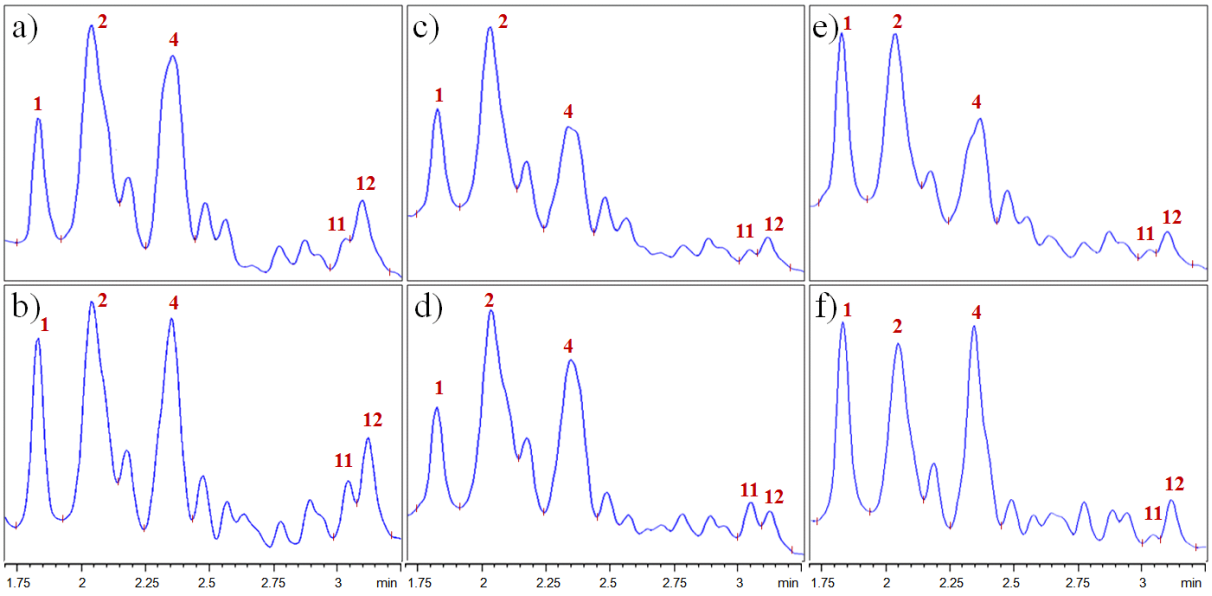


Figure 22: Chromatograms of HPLC C18 separation of individual barley grain varieties: a) Jersey; b) Tolar; c) Blaník; d) Bojos; e) Malz; f) AF Lucius. The areas of labelled peaks were used for the varieties comparison.

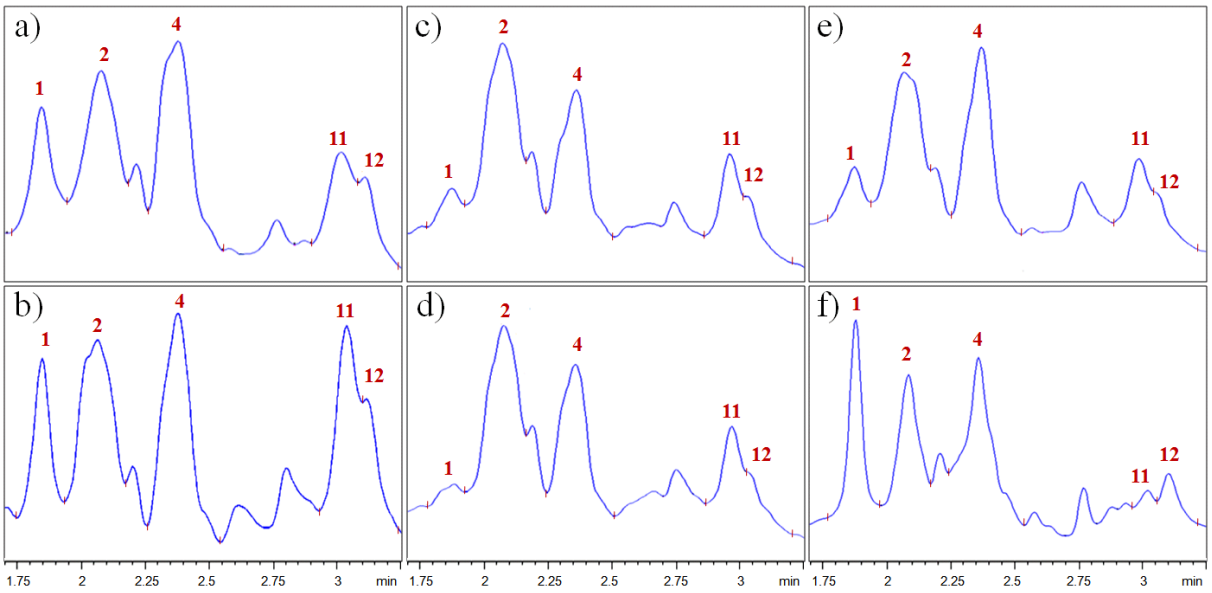


Figure 23: Chromatograms of HPLC C18 separation of individual barley malt varieties: a) Jersey; b) Tolar; c) Blaník; d) Bojos; e) Malz; f) AF Lucius. The areas of labelled peaks were used for the varieties comparison.

Significant differences between some varieties were evident from obtained chromatograms. However, the comparison of individual varieties using chromatograms only was difficult. Therefore, five major HPLC peaks were selected (peaks No. 1, 2, 4, 11 and 12) and used for varieties comparison. The proportional representation of these peak areas (obtained by integration using ChemStation software) related to peak No. 2 was performed for each variety and expressed graphically in Figure 24.

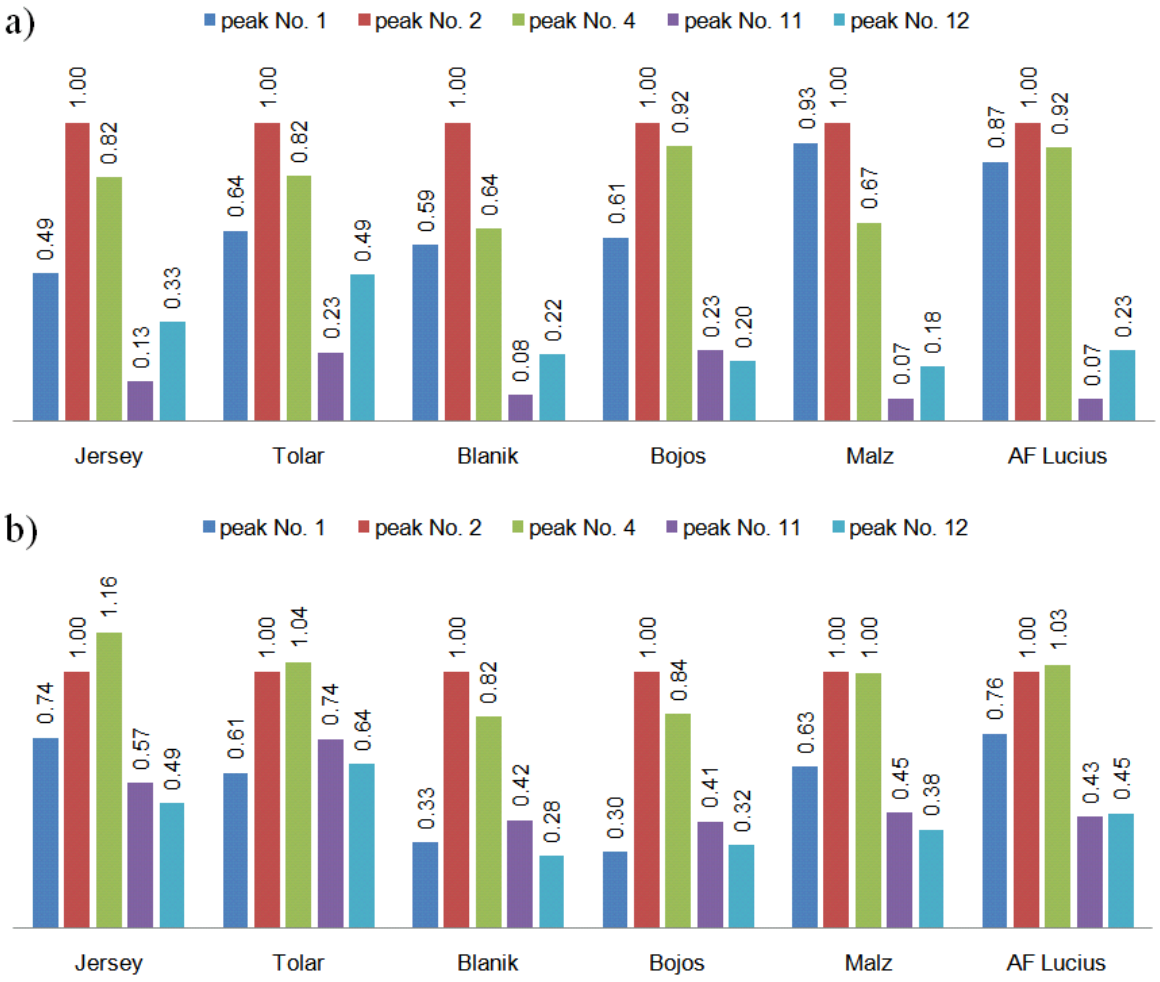


Figure 24: The proportional representation of five major peak areas related to peak No. 2. Individual barley varieties of a) grain; and b) malt were compared.

When comparing barley grains proportional representation profiles of individual varieties, the non-malting variety AF Lucius differed the most from others. This variety showed almost the same areas of first three major peaks. Next, only Malz variety showed the larger area of peak No. 1 than in peak No. 3. Varieties Jersey and Tolar showed similar profiles, and large areas of last peak (corresponding to protein Z; see Table 11, Chapter 5.1.5).

Even greater differences were evident from the malt profiles of individual varieties. Variety Tolar showed very large areas of peaks No. 11 and 12 (peak No. 11 represented two proteins in barley malt, namely β-glucosidase and β-amylase). Blanik, Bojos and Malz varieties had in common the very small area of the first peak. Moreover, Blaník and Bojos showed very similar spectra. In the AF Lucius variety profile, the smallest differences between individual peaks areas were evident. When comparing the chromatogram of this variety to all the others, the differences in the time range from 2.75 to 3 min were obvious. Some additional peaks were visible in this range in the chromatogram of AF Lucius.

For the determination of the reproducibility of this method, more batches of Tolar grain were analyzed as well. All these extracts were analyzed in different times. Four different extracts were used: three samples (sample 1-3) were extracted from one sample of milled barley grain (Tolar 1), and one sample (sample 4) was extracted from another sample of milled barley grain of the same variety (Tolar 2). The proportional representations of five

major peaks related to peak No. 2 were calculated and expressed graphically in Figure 25. The profiles of Tolar grains showed good long-term reproducibility. The standard deviations of individual peak representations varied from 0.01 to 0.05.

The greatest advantages of this method using HPLC separation of barley grain and malt aqueous extracts are its rapidity and simplicity. The comparison of C18 HPLC profiles could possibly be used for barley varieties discrimination. For this purpose, however, this method would need more optimization steps and more analyses should be performed. For example, the analysis of more barley varieties of grain and malt, the analysis of all of these samples in more batches, or the comparison of hordein profiles can be performed.

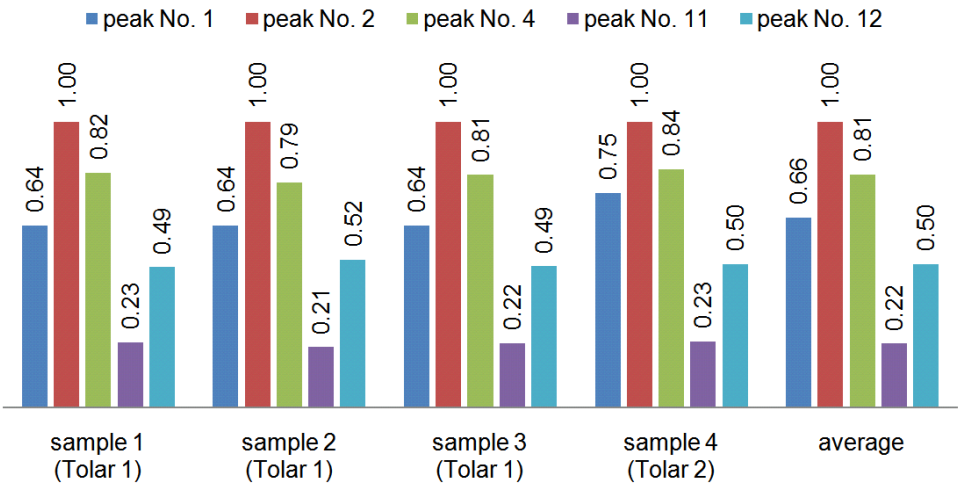


Figure 25: The reproducibility of C18 HPLC separation of Tolar grains.

5.4.2. Differences in low-molecular weight intact water-soluble proteins profiles of selected barley varieties

The aim of this study was to examine the differences in low-molecular weight water-soluble proteins profile of three different barley varieties: Jersey (Dutch well-proven malting variety), Tolar (Czech malting variety allowed for the production of Czech beer) and AF Lucius (non-malting variety). Grain and malt proteins of selected barley varieties were purified using Nanosep 3k centrifugal devices and analyzed by MALDI-TOF MS operated in linear mode.

Obtained spectra are shown in Figure 26. No significant differences were observed between the MS spectra of Jersey and Tolar grains extracts. In AF Lucius grain spectrum, the peak of 8.80 kDa showed higher intensity than peaks corresponding to LTP2 and LTP1b (7.10 kDa and 9.98 kDa, respectivelly), which is in contrast to other two varieties. This peak is present also in malt of AF Lucius variety, while it was not detected in Jersey and Tolar malt. The glycation of LTP1b protein was observed in the Jersey and Tolar malt spectrum. Four glycated forms of LTP1b were detected in Jersey malt spectrum, and three glycated forms of LTP1b were detected in Tolar malt spectrum. Conversely, only one glycated form of LTP1b slightly appeared in AF Lucius malt spectrum. Also protein Z fragment showed lower intensity in AF Lucius spectrum in comparison to Jersey and Tolar spectrum, and the glycation of protein Z fragment was not detected in the AF Lucius malt spectrum. Glycated barley proteins are important for good malting and brewing process, as well as for the quality of beer and beer foam.

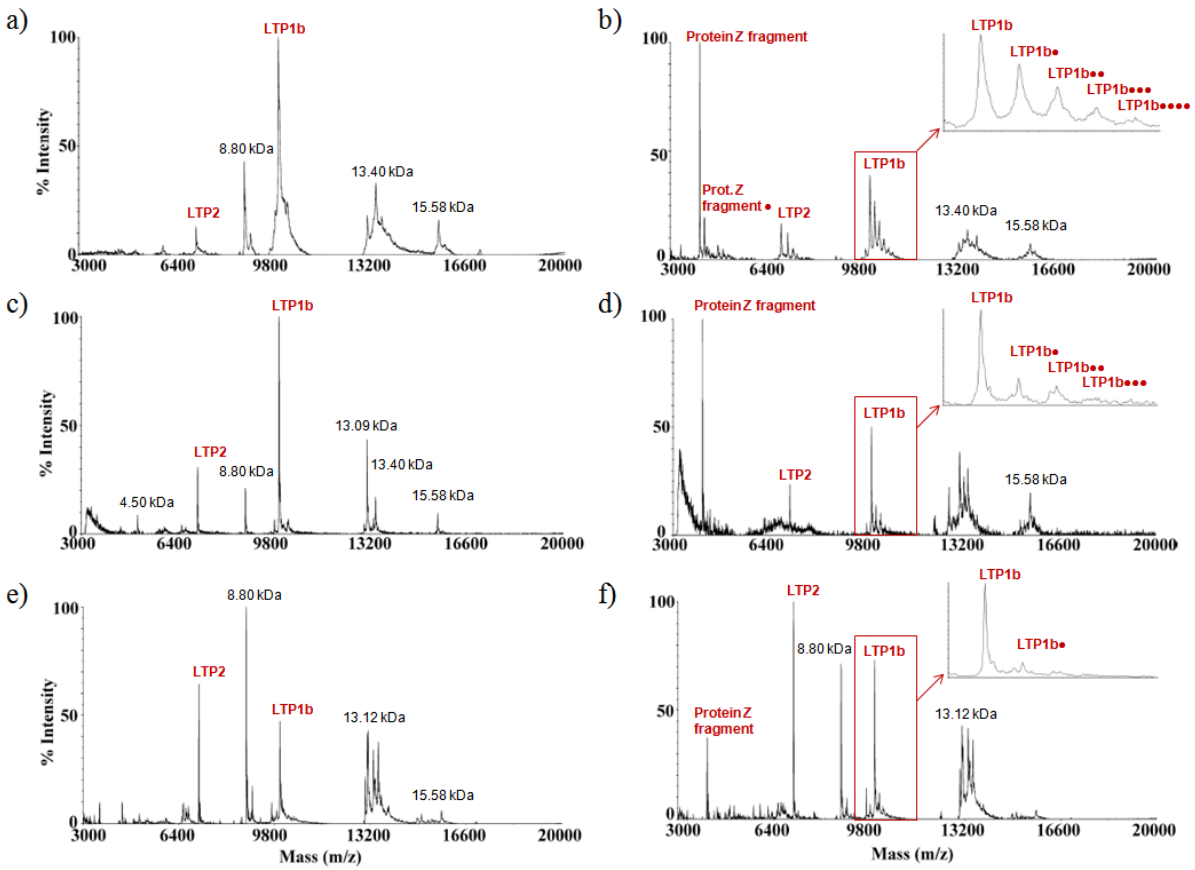


Figure 26: MALDI-TOF linear mode MS spectra of low-molecular weight grain and malt proteins of three different varieties: a) Jersey grain; b) Jersey malt; c) Tolar grain; d) Tolar malt; e) AF Lucius grain; f) AF Lucius malt. The non-enzymatic glycation by one hexose unit is marked by a dot sign (●).[100]

From obtained results it can be concluded that used method seems to be suitable for distinction between malting and non-malting variety of barley malt, and for selection of barley varieties suitable for the malting industry. These results were published in 2010 in the Journal of the Institute of Brewing.[100]

5.5. Non-enzymatic glycation of barley protein

The large amount of D-glucose released from the starch degradation during the malting process causes the glycation of some barley proteins, including protein Z, LTP1 or LTP2.[8,27,35,51,52] This non-enzymatic modification might prevent precipitation and unfolding of protein Z and LTP1 during the wort boiling, which affect the presence of these proteins in beer and allows the influence of the beer quality. Moreover, glycation of LTP1 and protein Z is associated with foam-promoting properties.[8,35,51] Therefore, the detailed investigation of barley proteins glycations was performed.

5.5.1. Glycation of low-molecular weight proteins

This study was published in 2010 in the Journal of the Institute of Brewing.[100] and follows the chapter 5.1.7. It was found, that linear mode of MALDI-TOF MS is suitable for revealing of glycation of low-molecular weight proteins and monitoring of this modification during the malting process. The ladders of the MS peaks in MALDI-TOF MS spectra differing of about 162 Da (Figure 17) indicate the protein glycation. Detailed view on glycation of proteins in the mass area from 3.2 to 20 kDa during individual stages of the malting process is shown in Figure 27 (variety Tolar). During the malting process, LTP1b and LTP2 forms were gradually glycated with up to three and two hexose units, respectively. The glycated forms were detected from the 3rd day of the malting process.

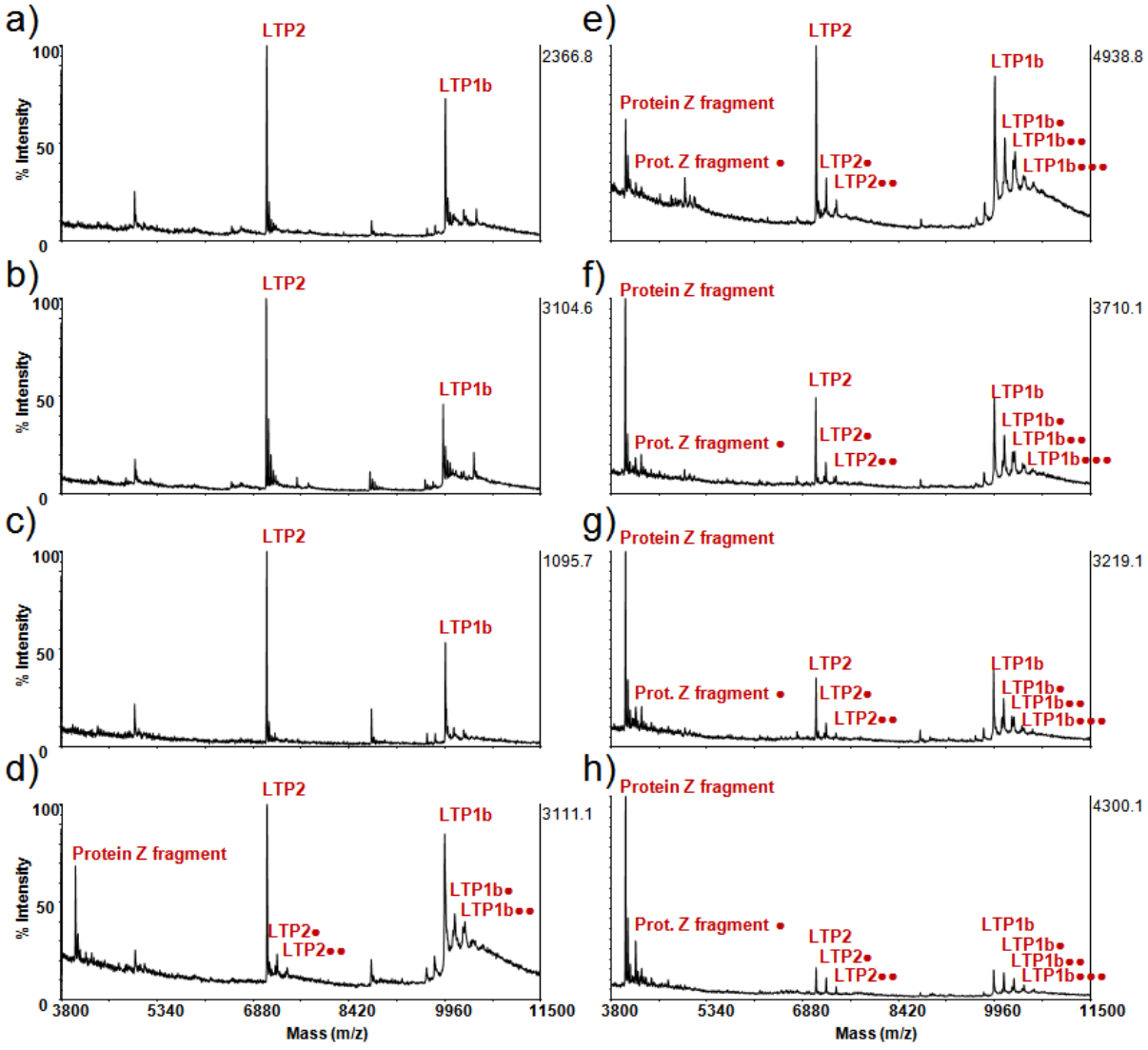


Figure 27: Linear mode MALDI-TOF MS spectra of low-molecular weight proteins from individual stages of the malting process: a) grain; b) 1st day of malting; c) 2nd day of malting; d) 3rd day of malting; e) 4th day of malting; f) 5th day of malting; g) green malt; h) malt. The non-enzymatic glycation by one hexose unit is marked by a dot sign (●).

Protein Z C-terminal 363 – 399 fragment was detected from the 3rd day of the malting process. The glycation of protein Z fragment with one hexose unit was slightly detected from the 4th day of the malting process. Glycated proteins are formed by reaction of free amino groups of lysine, arginine or N-terminal amino acid residues.[25,27] The amino acid sequence of protein Z obtained from UniProtKB database[101] (entry P06293) is shown in Figure 28. From the amino acid sequence of protein Z fragment (highlighted by blue colour) it can be assumed that this fragment may be glycated via amino group of Arg_{379} or N-terminal Val_{363} residue.

```
        10         20         30         40         50         60
MATTLATDVR LSIAHQTRFA LRLRSAISSN PERAAGNVAF SPLSLHVALS LITAGAAATR
        70         80         90        100        110        120
DQLVAILGDG GAGDAKELNA LAEQVVQFVL ANESSTGGPR IAFANGIFVD ASLSLKPSFE
       130        140        150        160        170        180
ELAVCQYKAK TQSVDFQHKT LEAVGQVNSW VEQVTTGLIK QILPPGSVDN TTKLILGNAL
       190        200        210        220        230        240
YFKGAWDQKF DESNTKCDSF HLLDGSSIQT QFMSSTKKQY ISSSDNLKVL KLPYAKGHDK
       250        260        270        280        290        300
RQFSMYILLP GAQDGLWSLA KRLSTEPEFI ENHIPKQTVE VGRFQLPKFK ISYQFEASSL
       310        320        330        340        350        360
LRALGLQLPF SEEADLSEMV DSSQGLEISH VFHKSFVEVN EEGTEAGAAT VAMGVAMSMP
       370        380        390
LKVDLVDFVA NHPFLFLIRE DIAGVVVFVG HVTNPLISA
```

Figure 28: The amino acid sequence of protein Z (UniProtKB entry P06293)[101]. Protein Z C-terminal 363 – 399 fragment is highlighted by blue colour and glycated Lys_{276} residue by red colour. Identified tryptic and chymotryptic peptides containing Lys_{276} residue are highlighted by dashed and full underline, respectively.

5.5.2. Glycation of protein Z

The glycation of proteins can be also detected by mass spectrometric fragmentation of glycated peptide. This study is focused on protein Z. After SDS-PAGE separation of barley grain and malt, bands corresponding to this protein were excised and protein Z was in-gel digested using two proteases – trypsin and chymotrypsin. Purified peptides were analyzed by MALDI-TOF/TOF MS.

Using tryptic digestion, a little bit higher coverage was obtained. Therefore, trypsin is slightly better enzyme of choice for identification of this kind of proteins. However, chymotrypsin was used in previous studies of protein glycation due to the trypsin cleavage specificity from C-terminal to Lys and Arg residues. These residues were expected to be glycated and thus resistant to tryptic hydrolysis of peptide bonds.[27,73]

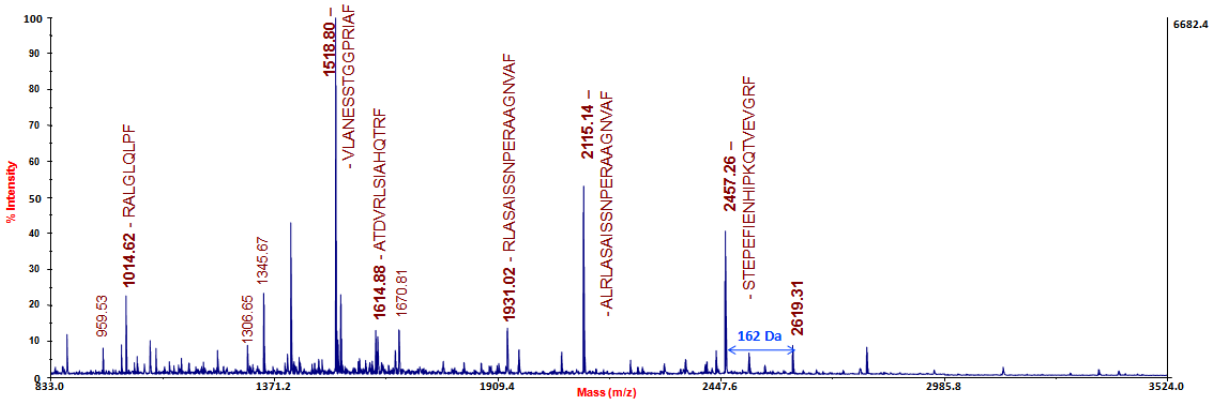


Figure 29: MALDI-TOF MS spectrum of barley malt sample. Bold highlighted peptides correspond to protein Z. The peptide of m/z 2619 represents a Lys-glycated protein Z peptide of m/z 2457 (S_{264}-F_{284}).

The MS spectrum of malt protein Z digested by chymotrypsin including identified peptides corresponding to protein Z (highlighted in bold) is shown in Figure 29. The peptide of *m/z* 2619 was not detected in barley grain and differs from protein Z peptide of *m/z* 2457 (S_{264}-F_{284}) about 162 Da, which indicated the glycation of this peptide. This suspicion was confirmed after MS/MS fragmentation of this peptide (Figure 30). Several b- and y- fragment ions, glycated y-fragment ions, immonium ion Lys–Hex[102], and the peptide ion fragment after loss of hexose were identified in the MS/MS spectrum. This peptide is supposed to be glycated via Lys_{276} residue.

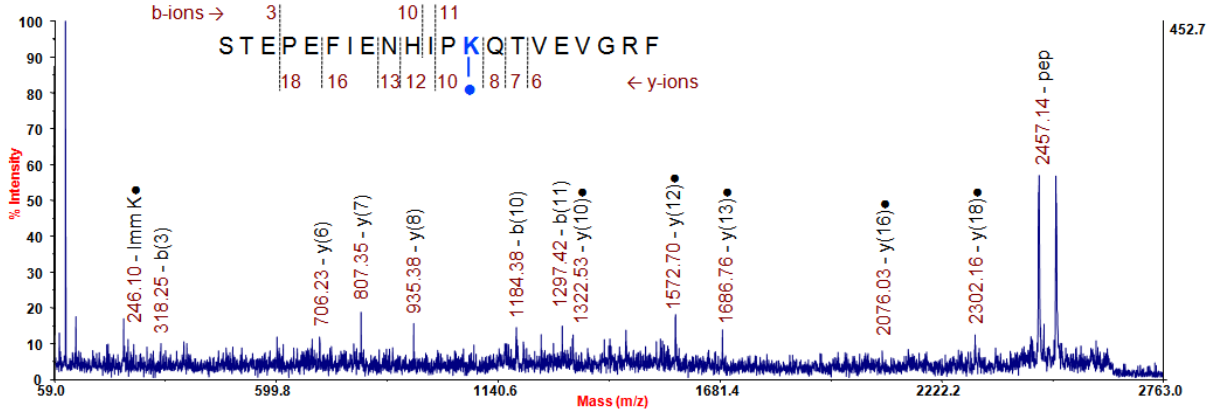


Figure 30: MALDI-TOF/TOF MS/MS fragmentation spectrum of non-enzymatically glycated peptide of m/z 2019. The glycation by one hexose unit is marked by a dot sign (●).

In addition, protein Z digested with trypsin was used for investigation of glycation using this method. The non-modified tryptic peptide of *m/z* 1652 (R_{262}-K_{276}), containing the potentially glycated Lys_{276} residue was identified in the barley malt sample. Nevertheless, no corresponding Lys-glycated peptide was detected. This confirms that glycated Lys residues are probably resistant to tryptic hydrolysis of peptide bonds.

MALDI-TOF MS is a suitable method for the analysis of non-enzymatically glycated proteins. The linear mode of TOF analyzer was successfully applied for the rapid monitoring of the level of low-molecular weight barley proteins glycation (namely ns-LTPs and fragment of protein Z) in individual steps of the malting process. However, this method is applicable only to low-molecular weight proteins because larger proteins hardly ionize by MALDI. The glycation of larger proteins can be analyzed after SDS-PAGE separation and chymotryptic digestion by the reflectron mode of MALDI-TOF MS. This method was successfully applied on analysis of glycated protein Z. The information about the proteins glycation is very important for maltsters and brewers because the glycation of barley proteins increase their stability, and therefore glycated proteins survive the malting and brewing process and positively influence the beer properties.[8]

5.6. Glycosylation of barley proteins

N-glycosylation represents the most frequently studied post-translational modification in plants. Study of barley glycoproteins is important for the agricultural and brewing industry due to impact of glycosylation on physicochemical properties and biological functions of proteins.[23,18,103] In addition, many plant glycoproteins are known to act as sensitizing agents in humans upon repeated exposure.[45]

Therefore, another task of this thesis was the investigation of barley glycoproteins. The enrichment of glycoproteins from a complex mixture is required for their analysis because modified proteins are often expressed with low abundance.[3,15] Barley water-soluble glycoproteins were analyzed after enrichment using one of the most well characterized and widely used lectin Concanavalin A (ConA) that binds to α-mannosyl and α-glucosyl residues.[72]

5.6.1. Glycoprotein enrichment using ConA lectin affinity column

Water-extracted proteins from barley grain and malt (variety Jersey) were separated by affinity chromatography on manually prepared columns packed with ConA-agarose. In comparison to previous studies, higher concentration of barley sample was chosen to obtain sufficiently concentrated minor bound glycosylated fraction in one affinity step. The sample was loaded in two steps to achieve perfect occupation of ConA binding sites. This process was optimized previously by Laštovičková in her doctoral thesis.[104]

Subsequently, bound and unbound protein fractions were separated by SDS-PAGE on the precast gradient gel. From gels of both barley grain and malt shown in Figure 31 it is evident that the majority of proteins occurred in the unbound fraction, and minor glycosylated proteins were found in the bound fraction. Individual proteins were excised, in-gel digested with trypsin, and identified by MALDI-TOF/TOF MS. Table 14 shows identified proteins in the bound grain and malt fractions. The ConA enrichment allows the identification of minor barley glycoproteins that were not able to be identified in the complex samples.

Preliminary results of this study were published in the journal Chromatographia in 2011.[106] However, some proteins remain unidentified even after numerous analyzes. Nevertheless, in February 2013, the obtained data were researched with the updated NCBInr database, resulting in the identification of additional proteins including several predicted proteins in both barley grain and malt (see Table 14). All identified proteins in both barley grain and

malt samples have at least one potential *N*-glycosylation site in their sequence, which means that some *N*-glycans can be attached to these Asn residue. Some of the barley grain and malt proteins identified in the bound fraction are even proven glycoproteins and/or allergens according to databases[101,105] (mentioned in the Table 14).

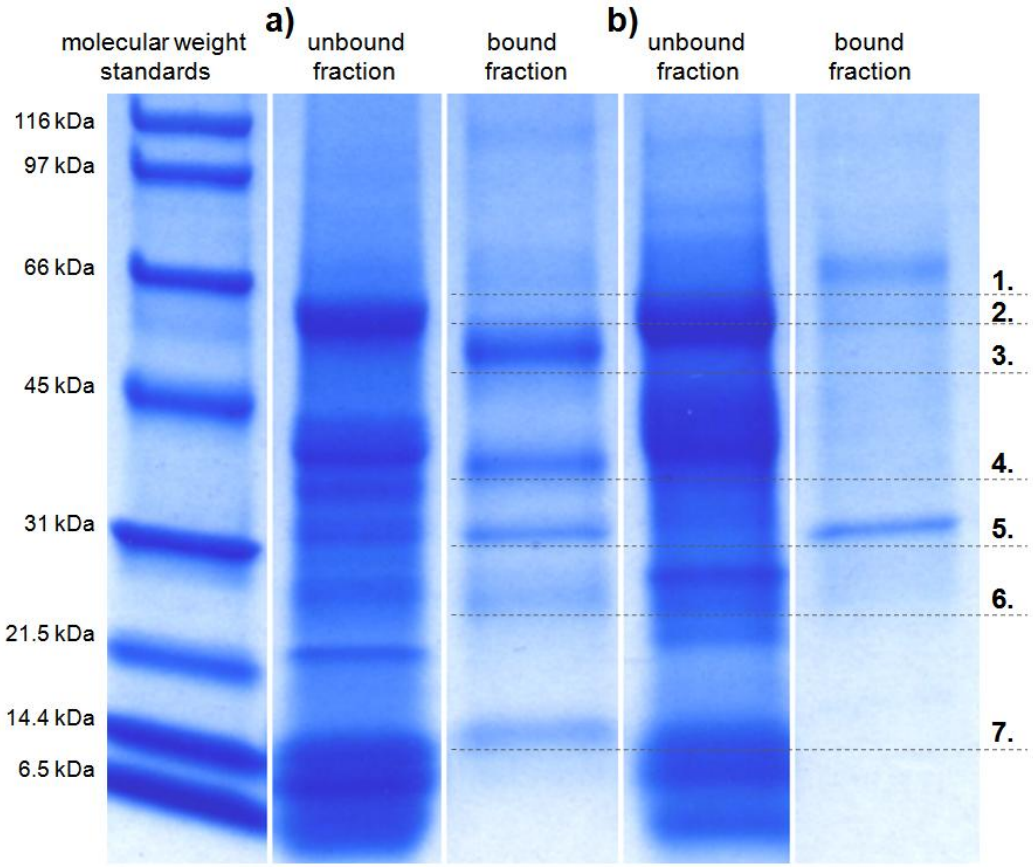


Figure 31: SDS-PAGE separation of the ConA AC unbound and bound fractions of barley a) grain; and b) malt. Protein bands from the bound fractions above the marked lines were analyzed.

Many barley malt and grain possible glycoproteins were identified after this separation method. Nevertheless, used manually prepared ConA column evinced one big problem. Concanavalin A was bleeding of the column and it resulted in occurrence of very intensive band in the SDS-PAGE gel that could overlay some important barley glycoproteins. Moreover, ConA was not detected only in this one band, but in the mass spectra of other bands as well (mainly in protein bands in the mass range of 10 – 40 kDa). The example of the identification of malt spot No. 7 is shown in the Figure 32. ConA is the predominant protein even in this band and the α-amylase inhibitor BMAI-1 was identified with the third highest score.

Therefore, we decided to find another column that would have the more firmly bound ConA resin. Successful results were obtained using the monolithic ConA HPLC column. This method is described in the next chapter.

Table 14: Proteins identified in barley grain and malt after ConA AC and SDS-PAGE separation. The molecular function of individual proteins[101,105] and the amino acids (AA) triplet of possible N-glycosylation sites (N-X-S/T; X ≠ P) ale listed in the table as well.

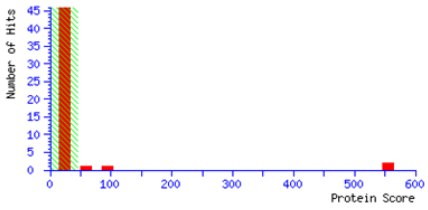
spot No.	grain	malt	UniProtKB entry	molecular function	possible *N*-glycosylation sites	
					quantity	position (AA triplet)
1.	predicted protein		F2DV95	peptidase activity	8x	92 (NDT); 157 (NAT); 217 (NVT); 360 (NLT); 366 (NET); 502 (NGT); 596 (NYS); 720 (NTS)
		beta-D-xylosidase	Q8W011	hydrolase activity, hydrolyzing O-glycosyl compounds	4x	203 (NSS); 432 (NAS); 473 (NVS); 710 (NAT)
	purple acid phosphatase isoform a		C4PKL2	acid phosphatase activity, metal ion binding	8x	140 (NYT); 205 (NTT); 236 (NGT); 292 (NKT; 414 (NYT); 465 (NFT); 500 (NET); 536 (NST)
2.	predicted protein		F2DJN8	alpha-mannosidase activity, carbohydrate binding, zinc ion binding	7x	30 (NTS); 58 (NNS); 273 (NVT); 465 (NIT); 475 (NFS); 523 (NAS); 727 (NKT)
3.	predicted protein		F2CYL7	nutrient reservoir activity	3x	228 (NTT); 371 (NLT); 405 (NGS)
		beta-amylase	Q9FUK6	beta-amylase activity, cation binding (allergenicity)	4x	237 (NDT); 249 (NGT); 338 (NFT); 402 (NQS)
4.	serine carboxypeptidase II		P08818	serine-type carboxypeptidase activity (glycoprotein)	7x	148 (NTS); 159 (NRT); 291 (NIS); 341 (NVT); 347 (NYT); 352 (NCS); 472 (NVT)
5.	concanavalin A		P02866	mannose binding, metal ion binding		
6.	germin B germin F germin D		Q9FYY4 Q9FYY3 Q9FYY2	manganese ion binding, nutrient reservoir activity	1x	79 (NVT) 79 (NVT) 78 (NVT)
7.	alpha-amylase inhibitor BMAI-1		P16968	alpha-amylase and serine-type endopeptidase inhibitor activity (glycoprotein, allergenicity)	1x	125 (NGT)

5.6.2. Glycoprotein enrichment using ConA HPLC column

To avoid the problems with bleeding of ConA from the column, glycoprotein enrichment using ProSwift ConA-1S monolithic HPLC column was performed. This column consists of polymeric monolith prepared by in-column polymerization, followed by functionalization with ConA. According to the manufacturer, this column and its HPLC compatibility provide high throughput, efficiency and accurate analysis.[72]

5.6.2.1. Optimization of glycoprotein enrichment using ConA HPLC column

The separation on ConA-1S monolithic HPLC column had to be optimized for sufficient separation of barley grain and malt samples. First, *N*-glycosylated and non-glycosylated protein standards were separated on this column for the efficiency testing, namely ribonuclease A (non-glycosylated protein) and ribonuclease B (RNase B). RNase B is glycoprotein containing high-mannose type *N*-glycans and frequently used for study of *N*-glycosylation. From chromatograms of these two standard proteins separations was evident that this column capture glycosylated protein.



1. gi|443370 **Mass:** 25583 **Score:** 555 **Matches:** 7(6) **Sequences:** 7(6) **emPAI:** 2.94
Chain A, High Resolution Crystallographic Studies Of Native Concanavalin A Using Rapid Laue Data Collection Methods
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
12	1095.7131	1094.7058	1094.5972	0.1087	0	80	1.7e-05	1		R.VGLSASTGLYK.E
27	1513.9172	1512.9099	1512.7460	0.1639	0	70	0.00016	1		K.ETNTILSWSFTSK.L
38	1573.0360	1572.0287	1571.8420	0.1867	1	105	7.7e-08	1		K.VGTAHIIYNSVDKR.L
72	2103.2957	2102.2884	2102.0491	0.2393	0	42	0.09	1		K.DLILQGDATTGTDGNLELTR.V
91	2590.6506	2589.6433	2589.3326	0.3107	1	129	1.3e-10	1		R.VGLSASTGLYKETNTILSWSFTSK.L
94	2832.7385	2831.7312	2831.3362	0.3950	0	76	2.6e-05	1		K.SPDSHPADGIAFFISNIDSSIPSGSTGR.L
105	3295.0920	3294.0847	3293.6092	0.4755	0	53	0.0042	1	U	R.LSAVVSYPNADSATVSYDVDLDNVLPEWVR.V

2. gi|72333 **Mass:** 25557 **Score:** 542 **Matches:** 7(5) **Sequences:** 7(5) **emPAI:** 2.24
concanavalin A - jack bean
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
12	1095.7131	1094.7058	1094.5972	0.1087	0	80	1.7e-05	1		R.VGLSASTGLYK.E
27	1513.9172	1512.9099	1512.7460	0.1639	0	70	0.00016	1		K.ETNTILSWSFTSK.L
38	1573.0360	1572.0287	1571.8420	0.1867	1	105	7.7e-08	1		K.VGTAHIIYNSVDKR.L
72	2103.2957	2102.2884	2102.0491	0.2393	0	42	0.09	1		K.DLILQGDATTGTDGNLELTR.V
91	2590.6506	2589.6433	2589.3326	0.3107	1	129	1.3e-10	1		R.VGLSASTGLYKETNTILSWSFTSK.L
94	2832.7385	2831.7312	2831.3362	0.3950	0	76	2.6e-05	1		K.SPDSHPADGIAFFISNIDSSIPSGSTGR.L
105	3295.0920	3294.0847	3293.6092	0.4755	0	40	0.08	2	U	R.LSAVVSYPNADATSVSYDVDLDNDVLPEWVR.V

3. gi|2506771 **Mass:** 15805 **Score:** 101 **Matches:** 2(2) **Sequences:** 2(2) **emPAI:** 0.87
RecName: Full=Alpha-amylase inhibitor BMAI-1; AltName: Full=Alpha-amylase flour inhibitor; AltName: Allergen=Hor v 1; Flags: Precursor
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
17	1206.7321	1205.7248	1205.5863	0.1385	0	56	0.005	1	U	K.ATVAEVFPGCR.T + Carbamidomethyl (C)
79	2218.3699	2217.3626	2217.1100	0.2527	1	45	0.041	1	U	K.ELGVALADDKATVAEVFPGCR.T + Carbamidomethyl (C)

Figure 32: The Mascot identification of malt spot No. 7 corresponding to α-amylase inhibitor BMAI-1, contaminated by ConA.

Afterwards, separation of barley grain and malt sample was optimized. Some separation conditions (namely composition of mobile phases and temperature) were set according to the column manual. Flow rate was set on 0.5 mL/min, when the column pressure was optimal and the separation time was acceptable. Concentration of the sample was set on 50 mg/mL at loop volume of 50 µL. At this concentration, the bound fraction showed sufficient intensity of detector response, especially when minor glycoproteins from barley grain were separated.

Most importantly, the optimization of the washing step period was performed to completely remove the unbound protein fraction. This optimization was accomplished using the malt sample and is shown in Figure 33. When the eluting mobile phase B was loaded at 2.3 minute, the unbound protein fraction was not completely removed (Figure 33a). Consequently, the washing step was greatly extended. The elution mobile phase was loaded at 13.5 min, which resulted in complete washing of the non-glycosylated proteins (Figure 33c); however, the washing step seemed to be needlessly long. Therefore, the optimal gradient of mobile phases was set as follows: 100 % of mobile phase A in 10 min, 100 % of mobile phase B from 10.5 to 20 min (Figure 33b).

Figure 34 shows the optimized chromatogram of barley malt. When the peak areas of the bound and unbound fractions were compared, only approximately 10 % of malt protein content and 3 % of grain protein content were detectable in the bound fraction at 214 nm.

This study was published in 2013 in the Journal of Liquid Chromatography & Related Technologies.[103]

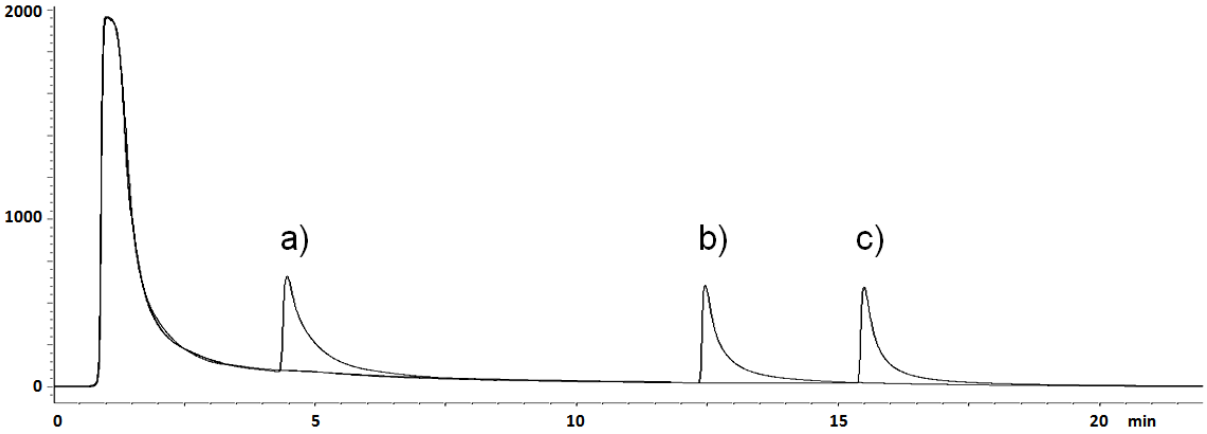


Figure 33: Overlaid HPLC chromatograms from the mobile phase gradient optimization process of ConA HPLC separation: a) mobile phase B was loaded at 2.5 min; b) mobile phase B was loaded at 10.5 min (gradient chosen for subsequent separation); and c) mobile phase B was loaded at 13.5 min. Mobile phase A contains microelements in Tris buffer, mobile phase B contains α-MMP in the mobile phase A. Very good reproducibility of this process was also evident from these chromatograms.[103]

5.6.2.2. Analysis of barley malt and grain glycoproteins

Water-soluble proteins extracted from barley grain and malt were separated on ConA-1S monolithic affinity HPLC column. Collected bound and unbound fractions were dialyzed, lyophilized and separated on linear gradient polyacrylamide gel 4 – 20 %. Individual bands of the ConA captured proteins were in-gel digested with chymotrypsin and after ZipTip C18 purification identified by MALDI-TOF/TOF MS.

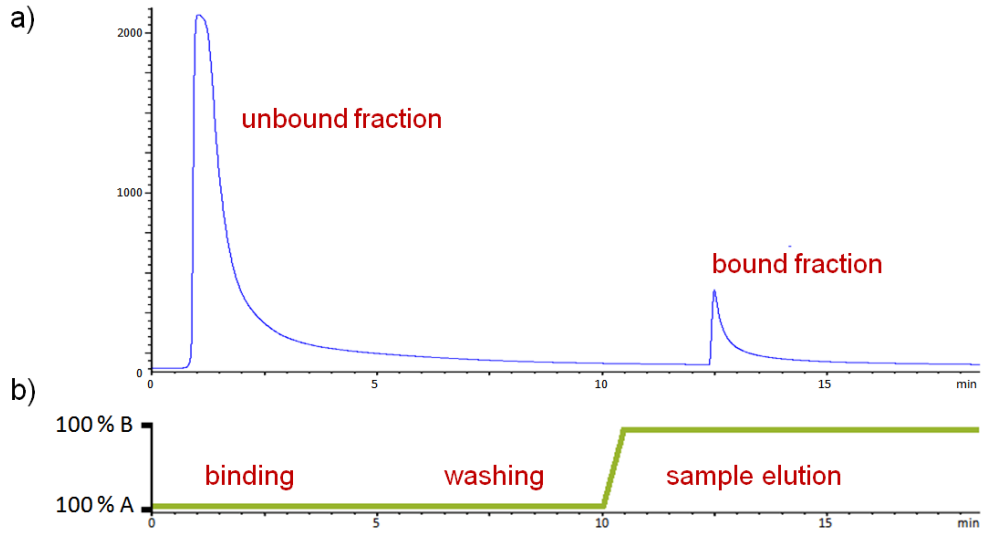


Figure 34: optimized HPLC ConA separation of barley malt: a) HPLC chromatogram; b) mobile phase gradient used for glycoproteins separation. Mobile phase A contained microelements in Tris buffer, mobile phase B contained α-MMP in the mobile phase A.[103]

With regard to further study of barley *N*-glycopeptides (chapter 5.6.4), a less specific chymotrypsin was used for ConA retained proteins digestion. It was found that tryptic digest will not give peptides of suitable size for study of glycopeptides. When some potential glycoproteins identified in barley grain or malt samples are theoretically digested with trypsin, only a few or no peptides containing the potential *N*-glycosylation site smaller than 3 kDa are created.[107] Chymotrypsin cleaves proteins of interest in smaller peptides, and therefore this enzyme was predominantly used for these studies.

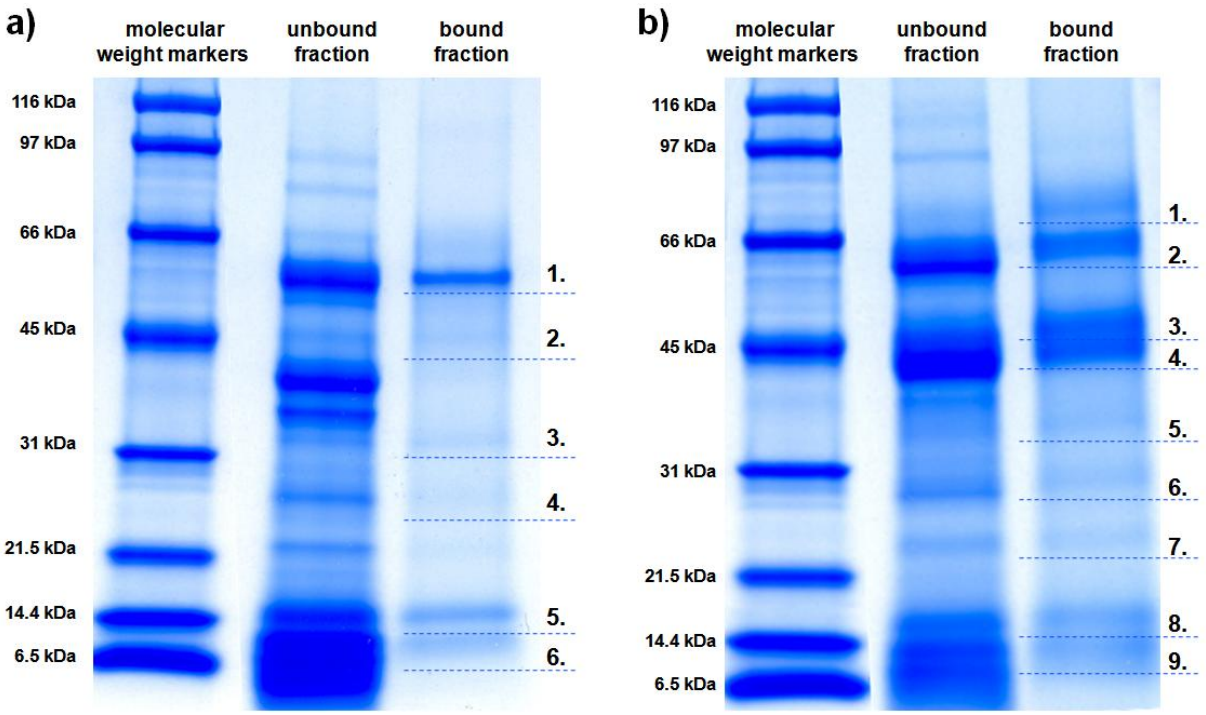


Figure 35: SDS-PAGE separation of the ConA HPLC unbound and bound fractions of barley a) grain; and b) malt. Protein bands from the bound fractions above the marked lines were analyzed.[103]

SDS-PAGE separations of barley grain and malt bound and unbound fractions are shown in Figure 35. Proteins identified in the bound fractions of barley grain and malt are summarized in the Tables 15 and 16, respectively. In barley grain, all identified proteins have at least one potential *N*-glycosylation site in their sequence. This fact means that all identified proteins in grain ConA bound fraction are possible *N*-glycoproteins. Nevertheless, the real possibility of *N*-glycosylation site occupancy is difficult to find out without knowing the crystallographic structure of the protein or without the study of glycopeptides or glycans after deglycosylation. The direct analysis of both glycan and peptide part is possible only when the glycan chain is relatively simple. But large glycan-substituted peptides are problematic to measure by MALDI-TOF/TOF MS because of their large mass and their tendency to be heterogeneous which results in peak broadening.[3] Moreover, non-glycosylated peptides interfere with ionization of glycopeptides and cause their considerable ion suppression.[85]

Table 15: Proteins identified in barley grain after HPLC ConA and SDS-PAGE separation. The molecular function of individual proteins[101,105] and the amino acids triplet of possible N-glycosylation sites (N-X-S/T; X ≠ P) ale listed in the table as well.

spot No.	name of identified protein	UniProtKB entry	molecular function	possible *N*-glycosylation sites	
				quantity	position (AA triplet)
1.	beta-glucosidase	Q40025	cation binding; hydrolase activity, hydrolyzing O-glycosyl compounds	3x	86 (NGT); 356 (NQT); 423 (NVS)
2.	predicted protein	F2E2X6	aspartic-type endopeptidase activity	4x	247 (NIT); 245 (NWT); 359 (NQT); 422 (NFT)
	predicted protein	F2DP98	serine-type carboxypeptidase activity	3x	45 (NSS); 187 (NPT); 267 (NVT)
3.	predicted protein	F2DIK1	unknown ferritin-like protein family	2x	85 (NLT); 151 (NTT)
4.	predicted protein	F2CYL7	nutrient reservoir activity	3x	228 (NTT); 371 (NLT); 405 (NGS)
5.	alpha-amylase inhibitor BMAI-1	P16968	alpha-amylase and serine-type endopeptidase inhibitor activity (glycoprotein; allergenicity)	1x	125 (NGT)
6.	alpha-amylase/trypsin inhibitor CMb	P32936	alpha-amylase and serine-type endopeptidase inhibitor activity (glycoprotein; allergenicity)	1x	124 (NLT)

In contrast to barley grain, not all proteins identified in the ConA retained malt fraction are possible *N*-glycoproteins. Considering the fact that ConA bounds mannosyl and glucosyl residues, also non-enzymatically glycated proteins that are highly created during the malting process may be probably captured by this column. Another possibility is retention of non-specifically bound proteins as the result of commonly occurring protein-protein interactions.[108]

Table 16: Proteins identified in barley malt after HPLC ConA and SDS-PAGE separation. The molecular function of individual proteins[101,105] and the amino acids triplet of possible N-glycosylation sites (N-X-S/T; X ≠ P) ale listed in the table as well.[103]

spot No.	name of identified protein	UniProtKB entry	molecular function	possible *N*-glycosylation sites	
				quantity	position (AA triplet)
1.	beta-D-xylosidase	Q8W011	hydrolase activity, hydrolyzing O-glycosyl compounds	4x	203 (NSS); 432 (NAS); 473 (NVS); 710 (NAT)
	predicted protein	F2DD64	hydrolase activity, hydrolyzing O-glycosyl compounds	6x	41 (NYT); 146 (NET); 462 (NAT); 521 (NMS); 539 (NQT), 660 (NFS)
2.	beta-amylase	P16098	beta-amylase activity; cation binding (allergenicity)	4x	237 (NDT); 249 (NGT); 338 (NFT); 402 (NQS)
	beta-glucosidase	Q40025	cation binding; hydrolase activity, hydrolyzing o-glucosyl compounds	3x	86 (NGT); 356 (NQT); 423 (NVS)
3.	alpha-amylase type B isozyme	P04063	alpha-amylase activity; cation binding	1x	372 (NES)
4.	protein z-type serpin	P06293	nutrient reservoir activity; serine-type endopeptidase inhibitor activity (allergenicity)	2x	93 (NES); 170 (NTT)

spot No.	name of identified protein	UniProtKB entry	molecular function	possible *N*-glycosylation sites	
				quantity	position (AA triplet)
5.	predicted protein	F2DIK1	unknown; ferritin-like protein family	2x	85 (NLT); 151 (NTT)
	serine carboxypeptidase I	P07519	serine-type carboxypeptidase activity (glycoprotein)	3x	148 (NVS); 262(NAT); 407 (NLT)
6.	predicted protein	F2EBM4	nutrient reservoir activity	5x	140 (NAT); 222 (NWT); 300 (NLT); 426 (NGS); 469 (NTT)
7.	chain C, Amy2BASI PROTEIN-protein complex from barley seed	P07596	alpha-amylase inhibitor activity; serine-type endopeptidase inhibitor activity	N/A	-
	cold-regulated protein	Q9FSI8	phosphatidylethanolamine-binding	1x	73 (NIS)
8.	alpha-amylase/trypsin inhibitor CMd	P11643	alpha-amylase inhibitor activity; serine-type endopeptidase inhibitor activity	N/A	-
	putative splicing factor 3b	A7Y0E4	nucleic acid binding	1x	235 (NET)
	alpha-amylase inhibitor BMAI-1	P16968	alpha-amylase and serine-type endopeptidase inhibitor activity (glycoprotein; allergenicity)	1x	125 (NGT)
9.	alpha-amylase/trypsin inhibitor CMb	P32936	alpha-amylase and serine-type endopeptidase inhibitor activity (glycoprotein, allergenicity)	1x	124 (NLT)
	alpha-amylase/trypsin inhibitor CMa	P28041	alpha-amylase and serine-type endopeptidase inhibitor activity (glycoprotein, allergenicity)	N/A	-

Some of the barley grain and malt proteins identified in the bound fraction are proven glycoproteins according to database[101] (mentioned in the Tables 15 and 16). Nevertheless, the glycosylation of the other proteins is not known yet, moreover, several identified proteins are still named "predicted proteins", especially in the grain sample. This implies that this area of barley minor glycoproteins is still little studied. The MS spectrum corresponding to predicted protein (UniProtKB entry F2DIK1, its amino acid sequence and MS/MS fragmentation of one peptide is shown in Figure 36.

Some of the identified proteins are also known to act as sensitizing agents according to database[105] (mentioned in the Tables 15 and 16). The *N*-linked glycans of plant and insect glycoproteins are the most abundant environmental immune determinants. The two main motifs in allergenic *N*-glycans are the xylose and the core-3-linked fucose.[46] In barley, most of the allergenic proteins characterized so far belong to the α-amylase/trypsin inhibitor family.[43] Both β(1-2) xylose and α(1-3) fucose have been found in glycosylated inhibitors BMAI-1 and CMb[45] that were identified in our study in ConA captured fraction of barley grain as well as malt.

The enrichment of glycoproteins on ConA HPLC column was successfully optimized. Thanks to this, several minor potential glycopeptides were identified in the barley grain and malt samples.

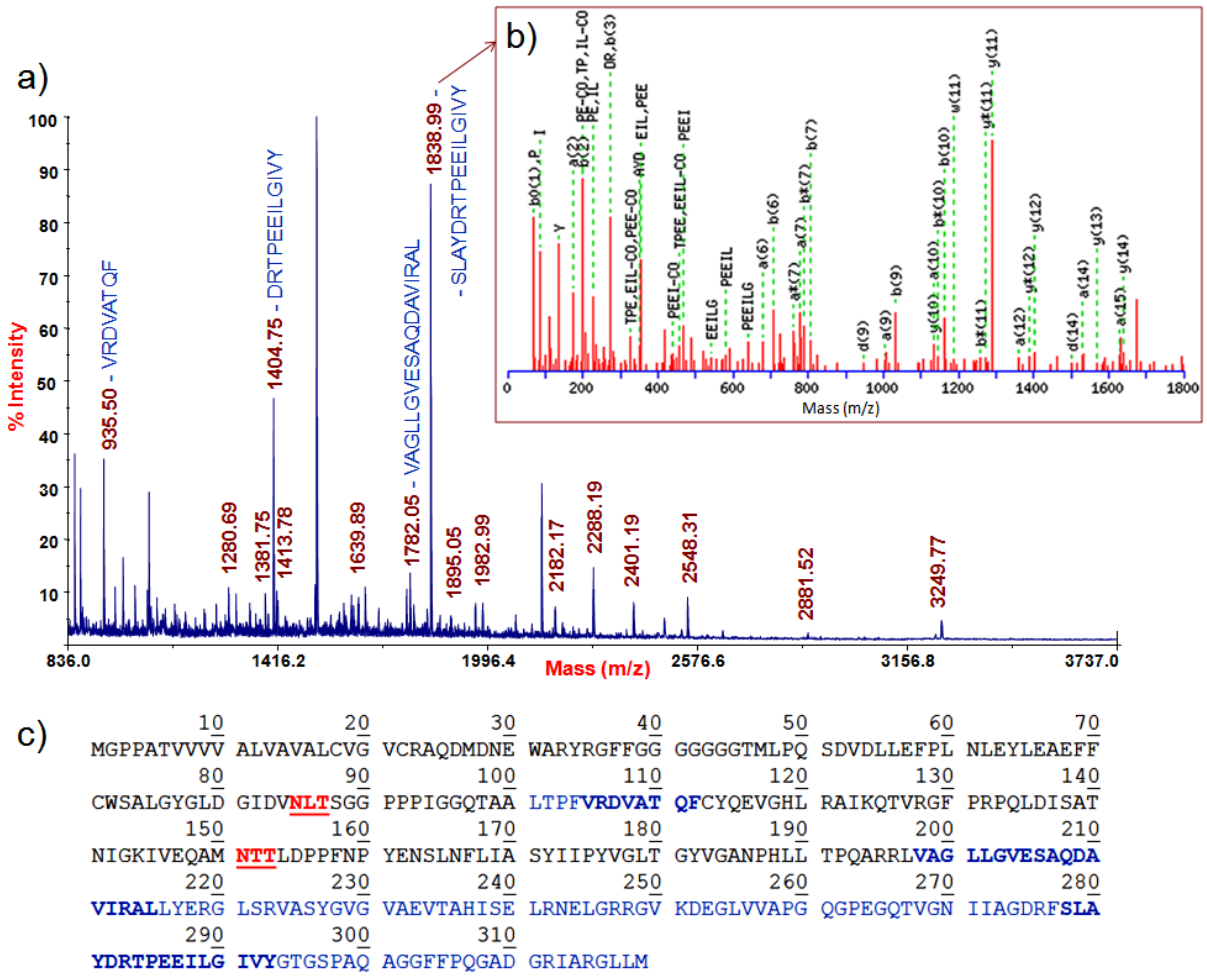


Figure 36: Illustration of MALDI-TOF/TOF MS identification of predicted protein (UniProtKB entry F2DIK1): a) MS spectrum with labelled identified peptides after PMF and MS/MS analysis; b) MS/MS fragmentation of peptide of m/z 1839 (S_{278}-Y_{293}); c) amino acid sequence with marked N-glycosylation potential sites (in red) and identified peptides (in blue) after PMF and MS/MS analysis (in bold).

In comparison to manually filled columns with ConA-agarose (chapter 5.6.1), ConA-1S monolithic column showed several improvements. As the most important one, the ConA stationary phase was firmly bound to the monolithic column. Therefore, the stationary phase was not washed easily and this column did not evince problem with ConA bleeding that caused complications when manually filled columns were used. This is very helpful for protein identification because ConA does not shield minor barley proteins with similar mass.

The main advantage of manually filled columns is their low cost purchase. Nevertheless, the glycoprotein enrichment using manually filled columns requires manual operations, especially for eluent loading and changing. The separation is time consuming because the flow rate is regulated by gravity only. Contrarily, the separation on ConA-1S column is faster due to the high pressure applied to the column (one separation, including column equilibration, took about 20 – 30 minutes), some operations are automated, and separated proteins are directly detected.

Moreover, Cona-1S column showed a very good reproducibility. The time of bound fraction elution remained still constant and therefore, using some suitable equipment, the whole process can be automated. Also, the profile of bound proteins on gel after SDS-PAGE separation remained identical, which was more difficult to achieve with the manually prepared columns.

This study was published in 2013 in the Journal of Liquid Chromatography & Related Technologies.[103]

5.6.3. Protein deglycosylation and glycan analysis

The most common approach in characterization of *N*-linked glycosylation involves the release of glycans from the isolated glycoprotein.[77] Protein deglycosylation can be achieved in a single step using several enzymes, for example PNGase F that hydrolyze the bond between the Asn side chain and the proximal GlcNAc of the oligosaccharide part.[23]

In this chapter, the optimization of deglycosylation process using standard glycoproteins, subsequent glycans purification and analysis using both MALDI-TOF and LC-ESI MS methods is described. Then, also barley grain and malt glycoproteins enriched by ConA HPLC and separated on SEC column were deglycosylated and analyzed using the optimized methods.

5.6.3.1. Optimization of protein deglycosylation process

The deglycosylation of glycoproteins using the enzyme PNGase F (from *Elizabethkingia meningosepticum*) was optimized using two model glycoproteins: RNase B, and ovalbumin. RNase B contains only high mannose-type *N*-glycans with the structure $GlcNAc_2Man_{5-9}$ attached to Asn_{60}. Contrary, *N*-linked glycans in ovalbumin consist of both hybrid-type and high-mannose-type oligosaccharides.[83]

With the aim to follow the used method of SDS-PAGE separation of ConA bound proteins, the in-gel deglycosylation method was optimized. Standard glycoproteins were separated by SDS-PAGE on 12% manually prepared gel and on precast linear gradient polyacrylamide gel 4 – 20 %. The glycoprotein bands were excised, and subsequently, reduced and alkylated proteins were in-gel deglycosylated using PNGase F. Three protein quantities were applied on the gel (7 μg, 3 μg and 1 μg) and the deglycosylation was performed using 6, 3 and 1 units of enzyme. However, no glycans were detected in either one sample after purification by C18 pipette tips and/or carbon tips (optimized purification methods are mentioned below).

Accordingly, we tried to in-solution deglycosylate the standard glycopeptides after SDS-PAGE and in-gel tryptic digestion. In the MALDI-TOF MS spectra, several peptides were detected. After peptides capture on C18 pipette tips, some peptides were still slightly visible and no glycans were detected. Even when samples were purified on carbon tips, no glycans were detected; also this procedure was not successful as well.

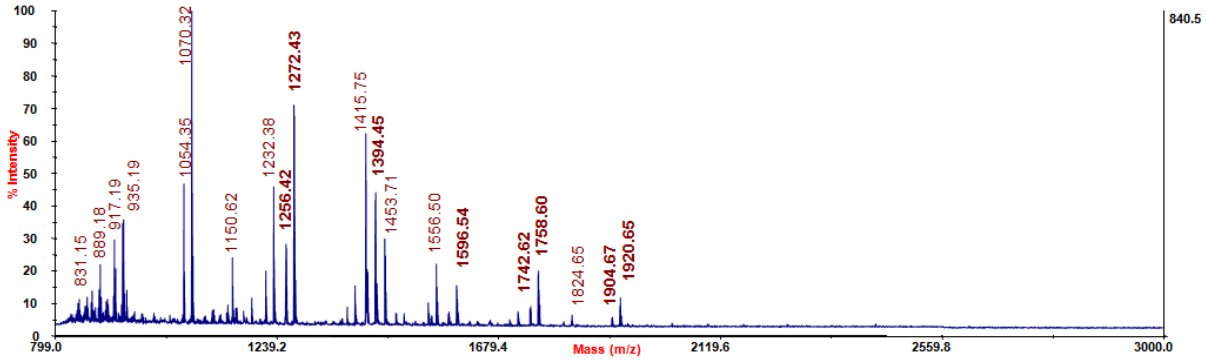


Figure 37: MALDI-TOF MS spectrum of deglycosylated RNase B sample without purification. RNase B glycans are highlighted in bold.

Therefore, the optimization of in-solution protein deglycosylation was performed. Standard glycoproteins were deglycosylated according to Laštovičková et al.[81] Even without purification, RNase B glycans were detected by MALDI-TOF MS, which confirmed the success of this method. (Figure 37) However, the intensity of glycans response in the MS spectra was low and some purification step was required. The purification of obtained glycans was optimized and all tested methods are described in the next chapter.

Table 17: Optimization of in-solution deglycosylation process using two standard glycoproteins: RNase B and ovalbumin.

Sample No.	glycoprotein (1 mg/mL)	DTT (50 mM)	PNGase F
1.	0.05 mg (50 µL)	50 µL	4 U
2.			2 U
3.			0.5 U
4.	0.01 mg (10 µL)	10 µL	1 U
5.			0.5 U
6.			0.2 U
7.	0.005 mg (5 µL)	5 µL	1 U
8.			0.5 U
9.			0.2 U
10.	0.001 mg (1 µL)	1 µL	0.5 U

In addition, the in-solution deglycosylation process was optimized using various combinations of protein and enzyme concentrations (Table 17). Regarding RNase B, majority of used concentrations combinations provided spectra with very good intensity. Only the sample No. 10 with protein concentration of 0.001 mg provided very poor MS spectra. Samples No. 7, 8 and 9 containing 0.005 mg of ovalbumin provided spectra with lower intensity than samples obtained from more concentrated sample. According to obtained results, PNGase F has high efficiency, and the high concentration of this enzyme was not needed for these standard glycoproteins. The minimal protein amount of 0.01 mg and 0.2 U of PNGase F seems to be suitable for successful glycan analysis for these types of samples.

5.6.3.2. Optimization of glycan purification

The glycan purification optimization was performed using samples of glycans released from 0.05 mg of RNase B. Sample purification using two types of carbon tips, Supel-Tips Carbon Micropipette Tips (maximum volume of 10 μL) and HyperSep Tips Hypercarb (maximum volume of 200 μL; Thermo Scientific, part of Thermo Fisher Scientific) was performed. Samples were diluted in water and slowly aspirated on activated tip and expelled back into the tube for approximately ten times. After water washing, bound glycans were eluted using 30% ACN. Using carbon Supel-Tips, satisfying results were achieved and the signal of glycans in MS spectra was improved. Contrary, after purification by HyperSep Tips, no glycans were detected. This may be caused by a large capacity of these tips together with low glycan concentration in the sample, or by different porosity of the carbon resin. Therefore, for further purification processes, carbon Supel-Tips were used.

In addition, deglycosylated protein was removed from the sample by capturing on C18 reversed phase to further improve the quality of the spectra. Again, two types of C18 pipette tips were tested, a tip with maximum volume of 10 μL (used for peptide purification), and a tip with maximum volume of 100 μL (OMIX tips, Varian, Walnut Creek, CA, USA). After purification using 10 μL C18 pipette tip, the capacity of the resin was insufficient and protein traces remained in the solution (measured by MALDI-TOF MS in linear mode using DHB/SA matrix). Majority of proteins were removed using 100 μl C18 pipette tips. However, the efficiency of both C18 pipette tips purification depends on the protein concentration in the sample.

For conclusion of this chapter, the successful results were obtained by combination of protein removal using C18 pipette tips and glycan desalting and purification using carbon pipette tips. After C18 purification, the sample dissolved in 0.1% TFA can be directly purified on carbon Supel-Tips. This purification method was used for all other samples.

The example of MALDI-TOF MS spectrum of purified RNase B glycans is shown in Figure 38. According to the literature, neutral carbohydrates ionize easily in MALDI-TOF MS positive ion mode under the formation of $[M + Na]^+$ as major ionic species, frequently accompanied by a less-abundant $[M + K]^+$ ion (where M represents the molecule of glycan).[14] These two species were observed in the MS spectra as a pair of peaks differing of about 16 Da. Without glycan purification (as well as after C18 purification only), the potassiated glycans $[M + K]^+$ were more intensive than the sodiated glycans $[M + Na]^+$ (see Figure 37). After carbon purification, the intensity of sodiated glycans increased (see Figure 38). The good intensity of sodiated glycans is important for successful MS/MS glycans fragmentation that is described in the chapter 5.6.3.4.

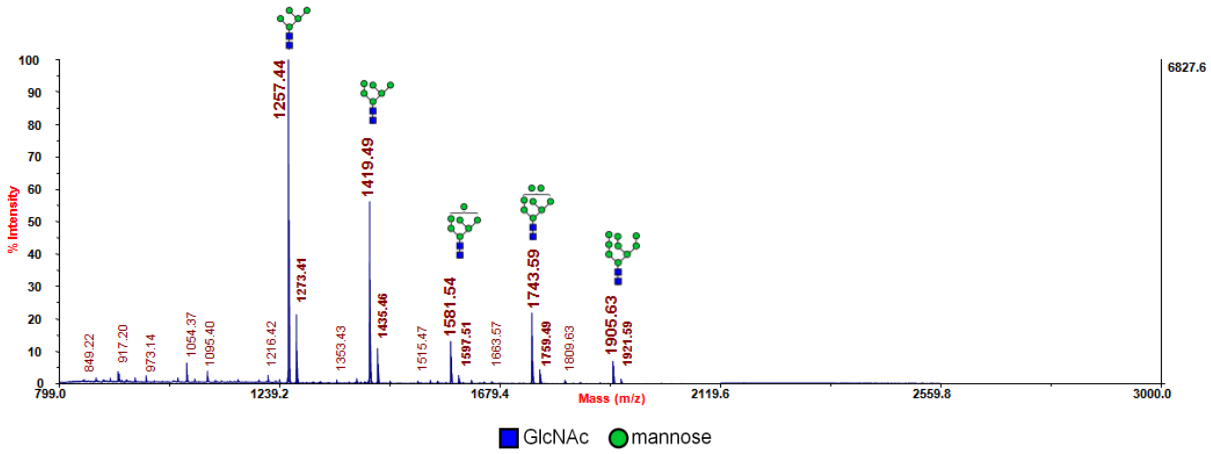


Figure 38: MALDI-TOF MS spectrum of RNase B glycans. Sodiated glycans (more intensive) and potassiated glycans differing of about 16 Da were detected and are highlighted in bold. The corresponding glycan structure is indicated as well.

5.6.3.3. The determination of suitable matrix for glycan analysis by MALDI-TOF MS

For the analysis of neutral carbohydrates, one of the first and still the most common matrices is 2,5-dihydroxybenzoic acid (DHB).[14] When the sample with DHB is crystallizing on the target, DHB tends to form large crystals at the periphery of the spot. The central region usually contains an amorphous mixture of sugar, contaminants, and salts. In order to produce a more homogeneous spot, the crystals may be recrystallized from ethanol to form a microcrystalline surface which improves the sensitivity of measurement.[14,109]

Therefore as the first choice of our experiments, various DHB concentrations and ACN concentrations in solvent were tested. The best results were obtained using the DHB concentration of 25 mg/mL in 40% ACN/0.1% TFA. Nevertheless, DHB crystallized in long needle-shaped crystals, which is in agreement with literature.[14] Since DHB spots were not homogenous, crystals needed to be manually targeted when MALDI-TOF MS analysis was performed. Moreover, one spot was suitable only for a few measurements because the sample amount was greatly reduced after laser ionization. After recrystallization in ethanol, DHB formed a homogenous spot; however, the sensitivity increase was not observed and the problem with rapid consumption of the sample remained.

Therefore, various MALDI matrices and binary matrices were tested for RNase B and ovalbumin glycans analysis. The 2-(4-hydroxyphenyl)azobenzoic acid (HABA) should give a fine crystalline surface and much more pronounced fragmentation.[109] The mixture of 2,4,6-trihydroxyacetophenone with diammonium citrate (THAP/DAC) is a suitable matrix for analysis of mixtures of neutral and acidic glycans in both positive and negative ion.[14] Using this matrix, Hao et al.[110] obtained a better result in the MS analysis of neutral glycans than using DHB matrix. THAP/DAC provided better signal-to-noise ratio, signal intensity and also better shot-to-shot and sample-to-sample reproducibility.[110]

According to these studies, THAP/DAC matrix was prepared by dissolving of 7.5 mg of THAP in 1 ml of ACN/20 mM ammonium citrate (1:1 v/v), and the HABA matrix was prepared in the concentration of 10 mg/ml in ACN/0.1% TFA 1:1, v/v. As the result, HABA matrix provided good MS spectra of RNase B glycans; however, ovalbumin glycans were not detected. The THAP/DAC matrix provided very poor spectra of RNase B and ovalbumin glycans.

To improve the crystallization of sample with DHB matrix and simultaneously preserve the good sample ionization properties of this matrix, DHB matrix was mixed in 1:1 (v/v) ratio with CHCA and SA matrix. DHB/CHCA binary matrix provided slightly weaker spectra, especially for ovalbumin glycans. DHB/SA binary matrix was a suitable matrix for these types of glycans. This matrix provided spectra with similar intensity to DHB. As an important improvement, small crystals were formed and the spot was homogenous. Moreover, this binary matrix allowed also an efficient analysis of intact low-molecular weight proteins in the linear mode of MALDI-TOF MS for the control of purification and deglycosylation process. Therefore, DHB matrix and DHB/SA binary matrix were used in further MALDI-TOF MS analyses of glycans.

5.6.3.4. Analysis of standard glycans by MALDI-TOF/TOF

The MS spectrum of RNase B glycans obtained after optimized purification is shown in Figure 38. DHB/SA was used as a matrix. In the spectrum, five major pairs of peaks are visible. Each pair of peaks differs by 16 Da, which represents the difference between sodium and potassium ion adducts. The sodiated glycans $[M + Na]^+$ are more intensive and correspond to five RNase B glycans ($GlcNAc_2Man_{5-9}$). All glycan ions were subjected to MS/MS CID fragmentation. Nevertheless, the potassiated glycans were poorly fragmented and only the fragmentation of sodiated glycans provided satisfying results. The illustration of MS/MS spectrum of RNase B is shown in Figure 39.

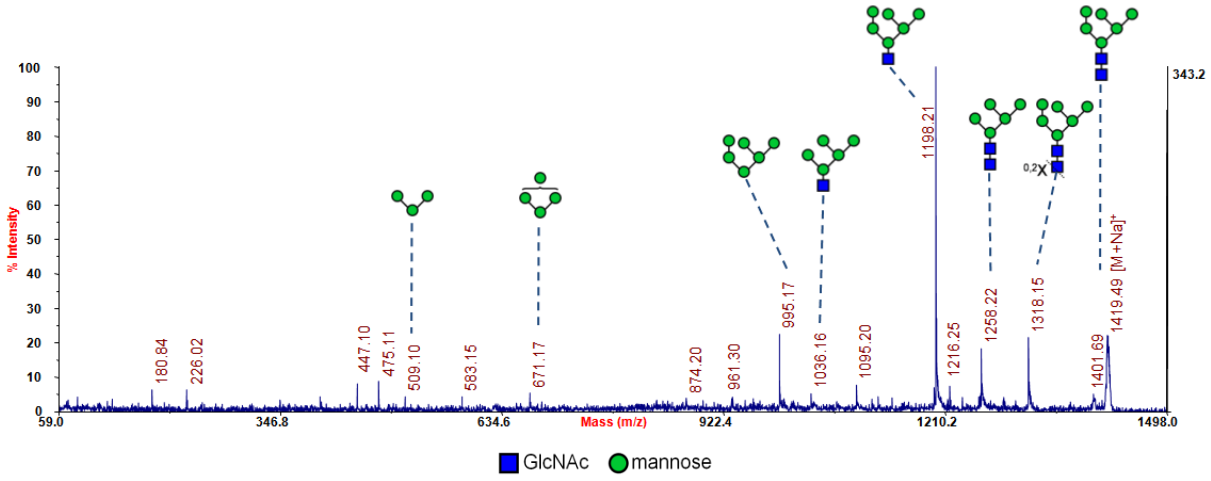


Figure 39: MALDI-TOF/TOF MS/MS fragmentation spectrum of RNase B glycan $GlcNAc_2Man_6$ (m/z 1419).

The MS spectrum of ovalbumin glycans is shown in Figure 40. DHB/SA was used as a matrix. Several glycans were detected in the MS spectrum and the glycan structures were assigned according to Harvey et al.[111] Nevertheless, the fragmentation of ovalbumin glycans resulted in poor fragmentation spectra that did not allow the identification of these glycans.

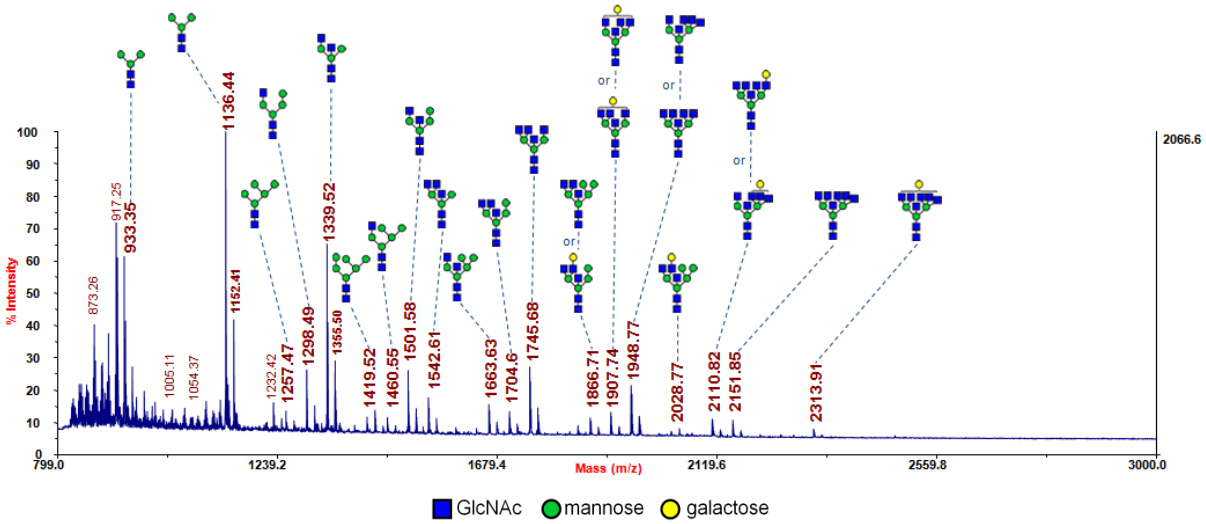


Figure 40: MALDI-TOF MS spectrum of ovalbumin purified glycans. The labelled glycan structures were assigned according to Harvey et al.[111]

5.6.3.5. Analysis of standard glycans using LC-ESI MS

Since the results from MALDI-TOF/TOF MS analysis of standard glycans were not fully convincing, glycans were analyzed using LC-ESI MS as well. Purified glycans were separated on Prevail Carbohydrate ES column in the following mobile phase's gradient: from 70% to 50% ACN in 5 min, followed by 50% ACN for 10 min. Separated glycans were detected in the positive mode using Esquire LC ion-trap mass spectrometer equipped with an ESI source.

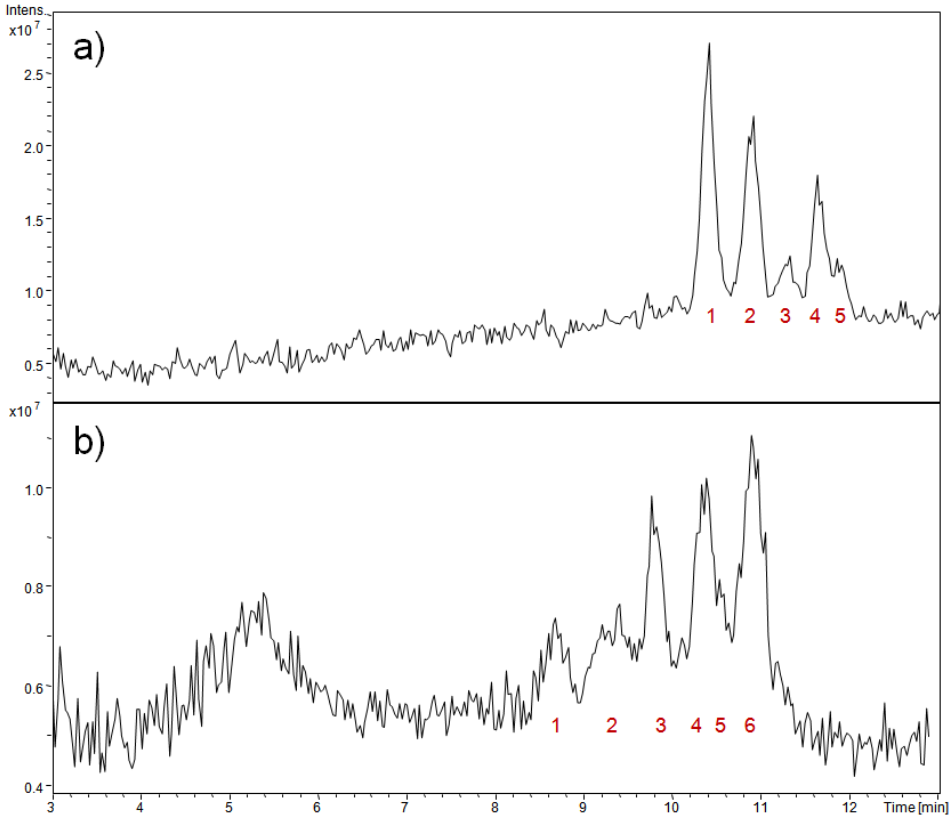
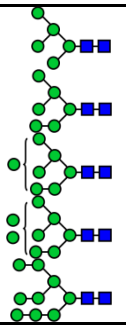
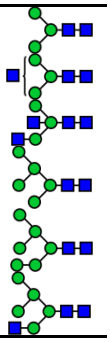


Figure 41: Ion chromatogram (total ion current) of: a) RNase B; b) ovalbumin.

The obtained ion chromatogram (total ion current) of standard samples is shown in Figure 41. Five signals were analyzed in RNase B and six signals in ovalbumin sample. When comparing both ion chromatograms, RNase B shows higher signal intensity. It may be caused for example by different sample concentration of by presence of contaminants. Glycans detected after ESI MS analysis are listed in the Table 18. The structures of ovalbumin glycans were assigned according to Harvey et al.[111]

Table 18: RNase B and ovalbumin glycans identified after LC-ESI MS. Structures of ovalbumin glycans were assigned according to Harvey et al.[111]

ribonuclease B	time [min]	$[M + Na]^+$	glycan structure	
1	10.3 – 10.5	1257.7	$GlcNAc_2Man_5$	
2	10.8 – 11.0	1419.7	$GlcNAc_2Man_6$	
3	11.1 – 11.4	1581.9	$GlcNAc_2Man_7$	
4	11.5 – 11.8	1744.0	$GlcNAc_2Man_8$	
5	11.8 – 12.0	1906.0	$GlcNAc_2Man_9$	
ovalbumin				
1	8.5 – 8.8	933.6	$GlcNAc_2Man_3$	
2	9.0 – 9.5	1136.6	$GlcNAc_2Man_3GlcNAc$	
3	9.7 – 9.9	1339.8	$GlcNAc_2Man_3GlcNAc_2$	
4	10.3 – 10.4	1258.8	$GlcNAc_2Man_5$	
5	10.8 – 10.9	1419.8	$GlcNAc_2Man_6$	
6	11.0 – 11.1	1460.9	$GlcNAc_2Man_5GlcNac$	

GlcNAc mannose

The ESI-MS/MS spectra of all glycans were measured. The example of fragmentation spectrum of high-mannose RNase B glycan (*m/z* 1419) is shown in Figure 42, and fragmentation spectrum of complex ovalbumin glycan (*m/z* 1339) is shown in Figure 43. The mass decrease of 18 Da indicated a loss of water molecule. The differences of 203 Da and 162 Da indicated the cleavage of GlcNAc and mannose residues, respectively. $^{0,2}X$ cleavages of GlcNAc residue were observed as well.

When comparing the analysis by MALDI-TOF MS and LC-ESI MS, LC-ESI MS resulted in better MS/MS glycan fragmentation and spectra with higher signal intensities were obtained. Nevertheless, larger number of ovalbumin glycans was detected by MALDI-TOF MS. Therefore, the optimal way to obtain the best result of glycan identification involves the combination of both MS methods.

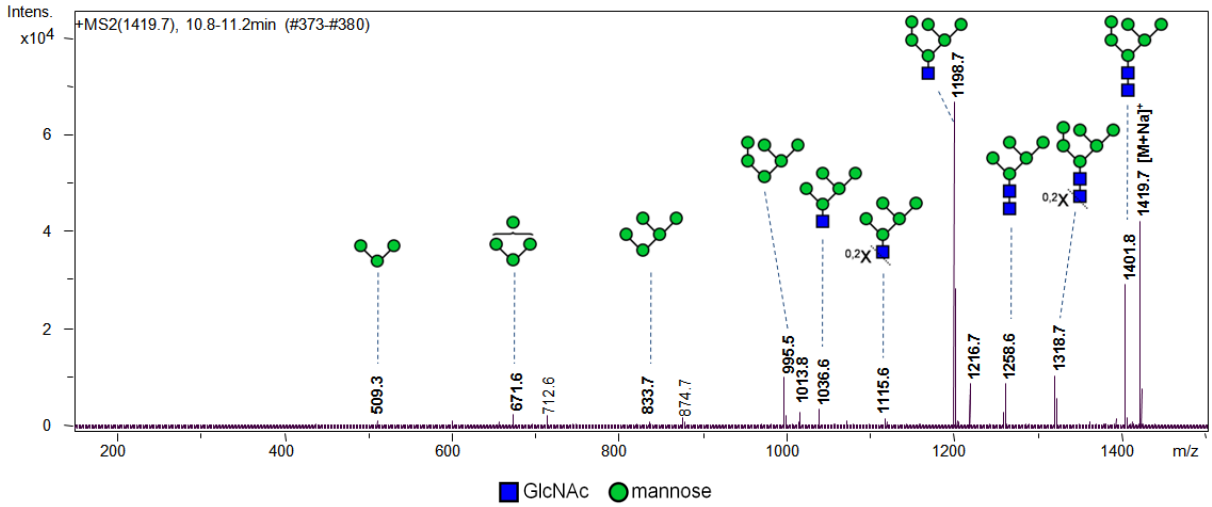


Figure 42: LC-ESI MS/MS fragmentation spectrum of RNase B glycan $GlcNAc_2Man_6$ (m/z 1419, 10.8 – 11.2 min).

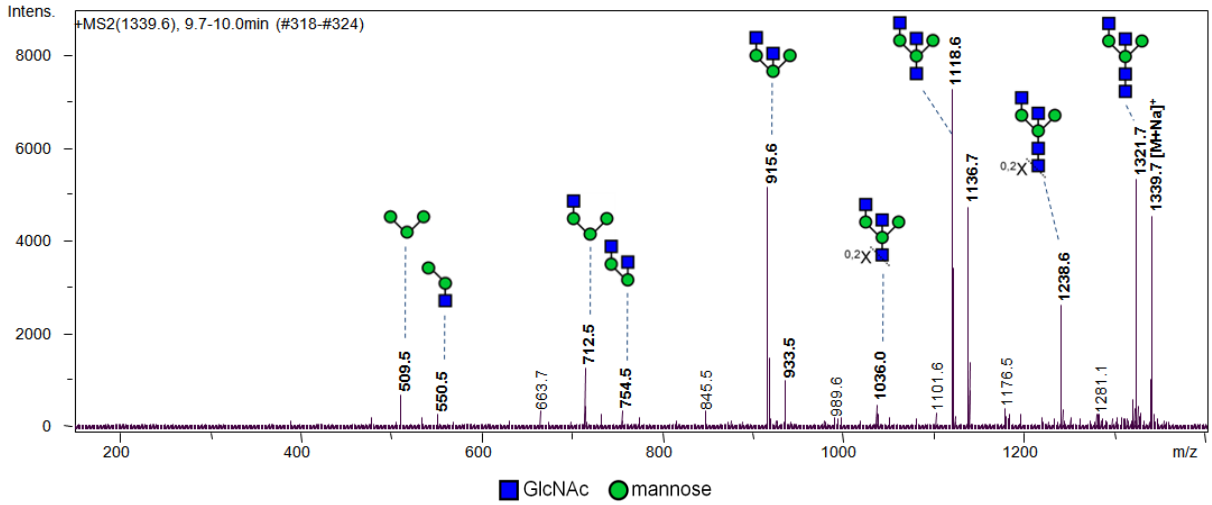


Figure 43: LC-ESI MS/MS fragmentation spectrum of ovalbumin glycan $GlcNAc_2Man_3GlcNAc_2$ (m/z 1339, 9.7 – 10.0 min).

5.6.3.6. Analysis of barley glycans by MALDI-TOF and LC-ESI MS

Optimized in-solution deglycosylation and glycan analysis methods were used for analysis of glycans from barley grain and malt glycoprotein fraction from HPLC ConA affinity column. Since these fractions contained several different proteins, it was more appropriate to separate the complex mixture before the deglycosylation process. Nevertheless, the well-established SDS-PAGE protein separation method used for analysis of these proteins could not be used in this study due to the negative results from the in-gel deglycosylation optimization method (chapter 5.6.3.1). Therefore, proteins should be separated still dissolved in a solution, for example by HPLC. We have chosen the SEC chromatography because proteins are separated according to their masses using this method similarly to SDS-PAGE. Barley grain and malt proteins were separated on eight fractions. Separated proteins were deglycosylated and glycans were purified and analyzed using the optimized methods. Samples were analyzed using both MALDI-TOF/TOF MS and LC-ESI MS.

Unfortunately, although some weak signals were detected in MALDI-TOF MS spectra of investigated grain and malt samples, no glycans were identified. The reason may be the small quantity of glycoproteins obtained after ConA HPLC separation and SEC HPLC fractionation. Another reason can be the specificity of used PNGase F enzyme. Although this enzyme exhibits specific activity for both high-mannose type and complex type *N*-glycans, it does not release *N*-glycans containing α(1-3) fucose residue linked to the proximal GlcNAc.[3] These types of glycans were found in some barley glycoproteins, that are associated with the allergy, namely the α-amylase inhibitors BMAI and CMb.[45] It is possible, that these core fucosylated *N*-glycans are present also in other barley glycoproteins.

Therefore in our next protein *N*-glycosylation studies we focused on the enrichment and analysis of glycopeptides, that allows the simultaneously analysis of both the glycan and peptide moiety and the distinction between core fucosylated and non-fucosylated *N*-glycans.

5.6.4. Enrichment and analysis of glycopeptides

The analysis of glycopeptides provides information about both peptide and glycan moiety. However, the analysis of glycopeptides in the complex mixture is almost impossible because glycopeptides hardly ionize in the presence of non-glycosylated peptides.[84,85] Therefore, protein *N*-glycosylation was studied after glycopeptides enrichment. First of all, the glycopeptides enrichment, purification and analysis were optimized using two standard glycoproteins. Then, barley glycopeptides were enriched using the optimized method from the various samples of grain and malt and the preliminary results are shown in the last chapter (0).

5.6.4.1. Optimization of glycopeptides enrichment and analysis

For this purpose, two standard glycoproteins were used. RNase B has already been used for glycan analysis and contains only high-mannose type glycans. The second used glycoprotein, horseradish peroxidase (HRP), is a plant glycoprotein that contains complex type glycans. It was found, that HRP glycans contain xylose and core 1,3-fucose, which had not been found in mammalian glycoproteins and are associated with the allergenicity of plant proteins.[46] Whereas the sequence of RNase B contains only one *N*-glycosylation site, the HRP sequence contains nine possible *N*-glycosylation sites. Amino acid sequences of these two standard glycoproteins including labelled *N*-glycosylation sites are shown in Figure 44.

As in the previous study of glycans (chapter 5.6.3.6), our first aim was to follow the study of barley glycoproteins separated by ConA HPLC and SDS-PAGE and enrich the glycopeptides from these samples of already known barley glycoproteins (chapters 5.6.1 and 5.6.2). Therefore, the optimization of glycopeptides enrichment using standard glycoproteins was performed after SDS-PAGE separation and in-gel digestion. Unfortunately, no glycosylated peptides were detected even after using of optimized procedure. Glycopeptides are probably tightly crosslinked in the gel and were not extracted from the gel. Following this, only in-solution protein digestion was used for the analysis of glycopeptides. For the glycopeptides enrichment optimization, both trypsin and chymotrypsin proteolytic enzymes were used.

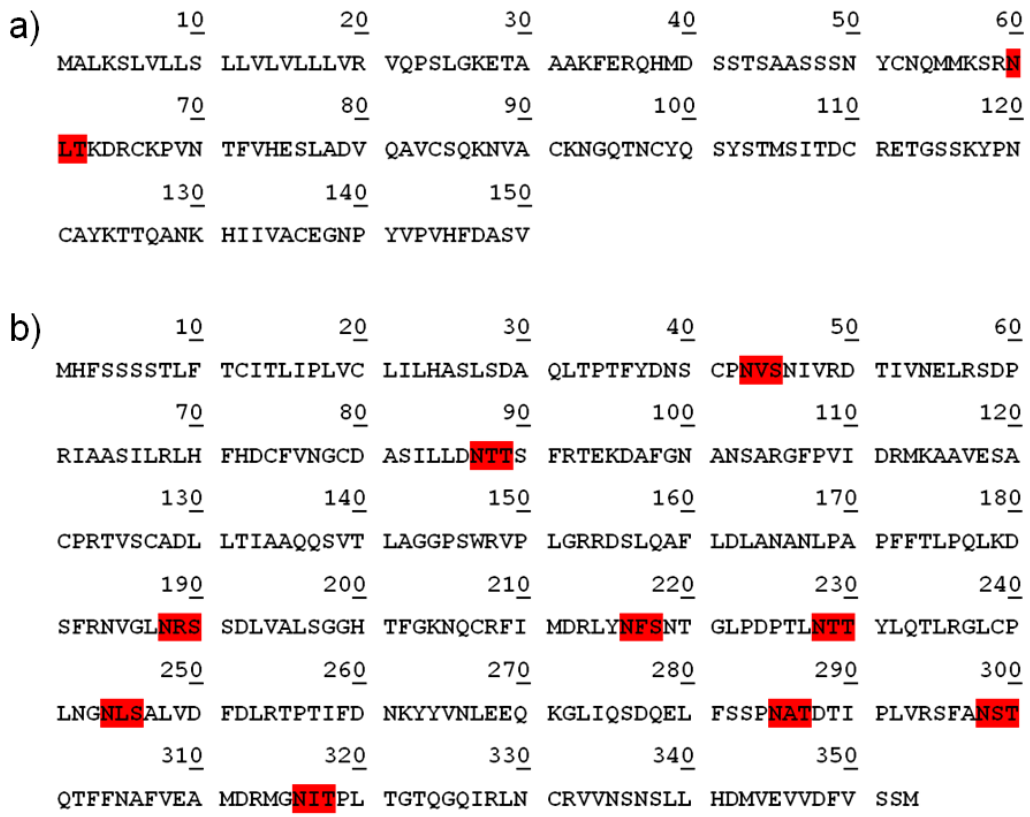


Figure 44: Amino acid sequences of a) RNase B); and b) horseradish peroxidase (HRP). Possible N-glycosylation sites are highlighted in red boxes.

Several glycopeptide analysis and enrichment strategies were tried, namely the MALDI-TOF/TOF analysis using glycopeptide-suitable matrices, and capture of glycopeptides on cellulose, HILIC or carbon resin. Nevertheless, all these methods did not bring satisfying results. For example, the cellulose column affinity chromatography was performed according to Kubota et al.[112] Cellulose TopTip was prepared using a GELoader tip filled with approximately 1 mg of cellulose. After MALDI-TOF MS analysis of the bound fraction it was found, that cellulose retains also non-glycosylated peptides, and among them, no glycosylated peptides were detected. Therefore, this application seems to be unsuitable for glycopeptides enrichment. The reason of failure could be using of different cellulose tips than Kubota et al. used.

The best results were obtained after glycopeptides enrichment on ConA lectin TopTips. Therefore, further analyzes were focused on the optimization of this enrichment strategy and the analysis of ConA captured glycopeptides. In contrast to C18 or carbon tips that are connected to pipette and thereby the sample is aspirated and expelled, sample is added using pipette on the ConA TopTip and then pushed through with syringe.

The glycopeptides enrichment was performed according to the manufacturer manual. ConA lectin captures glycopeptides containing mannosyl or glucosyl residues. Sample was slowly pushed through the tip for several times for proper glycopeptide binding. Then, unbound non-glycosylated peptides were washed from the resin, and glycosylated peptides were eluted by elution buffer containing glucose. The obtained glycopeptides are contaminated by salts and glucose from the elution buffer and therefore, the sample has to be purified before the MALDI-TOF/TOF analysis. After C18 ZipTip pipette tips purification, which was

successfully applied for peptide purification and is used also for glycopeptide purification[85], no glycopeptides were detected. Thus, glycopeptides are probably more hydrophilic than non-glycosylated peptides and were not retained on the C18 resin.

A successful purification of ConA captured glycopeptides was obtained using carbon Supel-Tips. After the purification, glycopeptides were analyzed by MALDI-TOF/TOF mass spectrometry.

For the mass spectrometric analysis of standard glycopeptides, several different matrices were tested. DHB matrix is the most commonly used matrix for glycopeptides analysis. According to Yu et al.[113], DHB matrix doped with 10 mM ammonium citrate was tested. Yu et al. have published that ammonium citrate improve the glycopeptide signal when added to DHB matrix solution, more specifically, they observed at least a 10-fold increase of ion intensity. The binary matrix of DHB/SA 1:1 that showed good results in study of glycans (chapter 5.6.3.3), was tried out as well. According to Franc et al.[114], ferulic acid (FA) was tested as a matrix suitable for analysis of *N*-glycopeptides. FA should have best result when large HRP or ovalbumin tryptic glycopeptides are measured.[114]

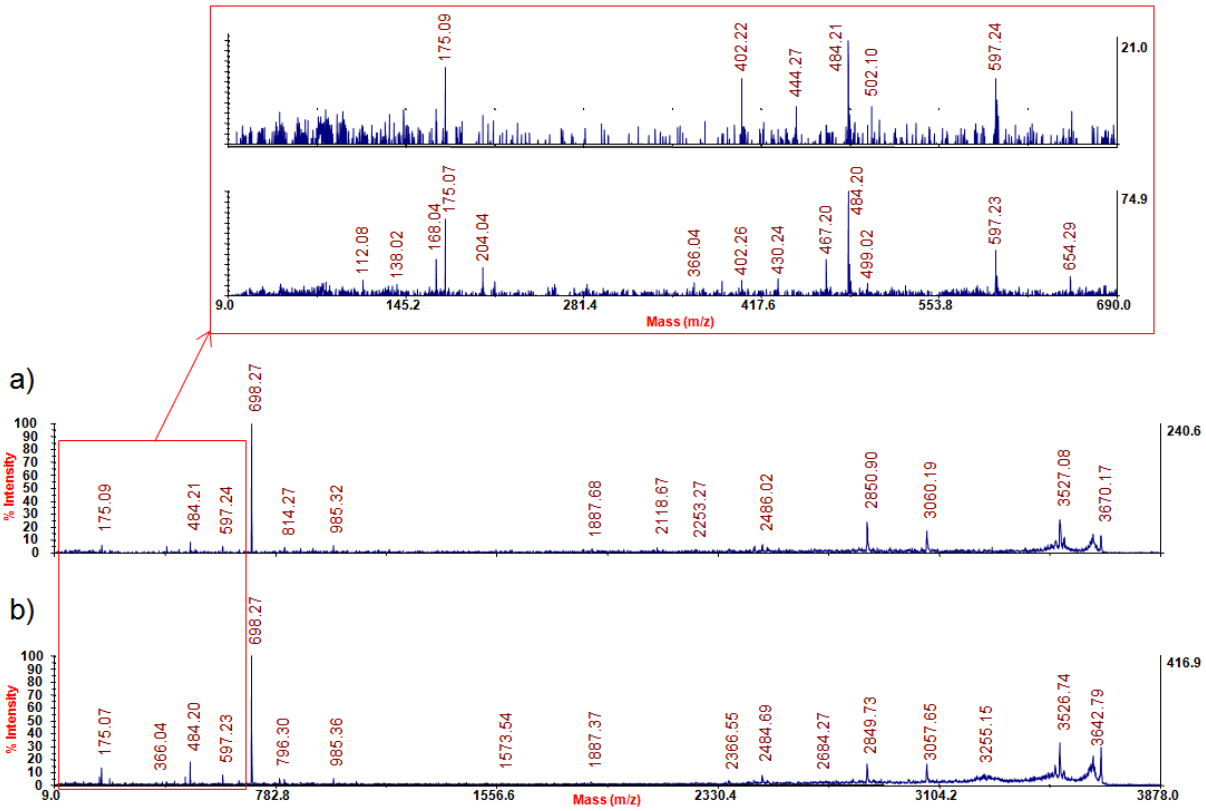


Figure 45: MALDI-TOF/TOF MS/MS fragmentation spectra of the tryptic glycopeptide from horseradish peroxidase (precursor at m/z 3670.9) obtained using a) DHB; and b) FA matrices.

DHB matrix provided the best results in MALDI-TOF MS and MS/MS analysis of standard *N*-glycopeptides. Nevertheless, this matrix formed large crystals and the spot was not homogeneously crystallized, and therefore, manually aiming at the crystals was necessary. But after recrystalization of the spot in ethanol the intensity of MS spectra decreased. Moreover, although the use of binary matrix DHB/SA forming small crystals brought good

results in analysis of glycans, poor spectra were obtained using DHB/SA matrix in analysis of glycopeptides. The reason may be the ability of peptides to desorb more easily from larger crystals than carbohydrates which are located within the inner microcrystalline region.[14] The ammonium citrate doped DHB matrix showed similar results as DHB matrix and no signal improvement was detected. FA showed weaker MS spectra than using DHB matrix in the case of RNase B glycopeptides; however, FA provided good results in the MS/MS fragmentation of large HRP glycopeptides (greater than about 3000 Da). The comparison between MS/MS spectra obtained using DHB and FA matrix is shown in Figure 45. The increase of signal intensity using FA matrix is evident. Especially when focus on the low-mass area, the N-acetylglucosamine (GlcNAc) oxonium ions (*m/z* 204 and 168), important for glycopeptide identification, were not detected using DHB matrix.

DHB matrix was preferably used for further analyzes of glycopeptides. As a supplement for measurement of large glycopeptides, samples were spotted also with FA matrix.

5.6.4.2. MALDI-TOF MS analysis of standard glycopeptides

Glycopeptides were analyzed by MALDI-TOF mass spectrometry and obtained MS/MS spectra were manually interpreted according to the literature (see chapter 2.4.1.3).[84] First, GlcNAc low-molecular-weight oxonium ions of *m/z* 204 and 186 and/or 168 were searched in MS/MS spectra. These oxonium ions are typical for fragmentation of glycopeptides in CID, and therefore, they may be considered as glycopeptides markers. Then, the characteristic fragment patterns for non-core-fucosylated or core-fucosylated *N*-glycopeptides (described in Figure 8) were searched. Thereby, the masses of both peptide and glycan moieties were determined. The structure of glycan moiety was identified according to glycan fragmentation signals, and the sequence of the peptide moiety was calculated according to the y- and b-ion fragment signals.

Both unbound and bound fractions from ConA glycopeptides enrichment were analyzed by MALDI-TOF MS and compared. The MS spectra of the unbound and bound fraction of tryptic-digested RNase B is shown in Figure 46. Significant differences between the peptide compositions in both samples were observed. Although some non-glycosylated peptides are still present in the bound fraction, several glycopeptides were detected. These glycopeptides did not occur in the unbound fraction at all. The presence of non-glycosylated peptides may be caused either by their incomplete washing, or non-glycosylated peptides may interact with glycopeptides by some specific or nonspecific forces.[85]

Figure 47 shows the zoomed MS spectra of RNase B tryptic glycopeptides including their identification obtained after MS/MS measuring. The glycan part of identified glycopeptides was composed of two GlcNAc units and five to nine mannose units. As a peptide part of RNase B glycopeptides, three different RNase B tryptic peptides were identified: the peptide with the sequence N_{60}-K_{63} of 475 Da, the peptide S_{58}-K_{63} (718 Da, created after one missed cleavage) and the peptide N_{60}-R_{65} (746 Da, created after one missed cleavage). The last mentioned one formed the most intensive glycopeptide signal in the MS spectra (*m/z* 1962.4) and all five *N*-glycosylation RNase B forms joined to this peptide were detected.

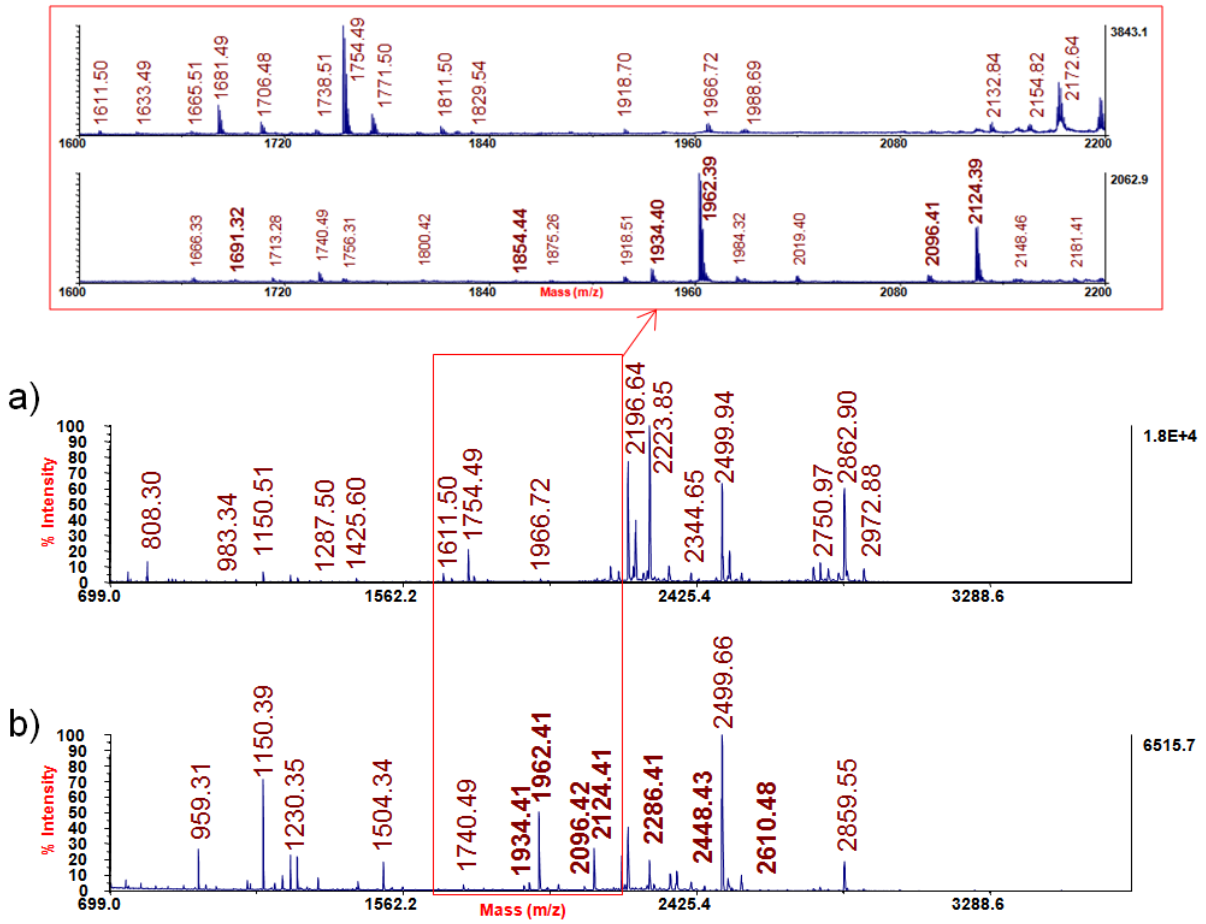


Figure 46: MALDI-TOF MS spectra of the a) unbound; and b) bound fraction from ConA TopTip RNase B tryptic glycopeptides enrichment. Identified glycopeptides are highlighted in bold. CHCA matrix was used in the case of ConA unbound peptides and DHB matrix for analysis of ConA bound glycopeptides.

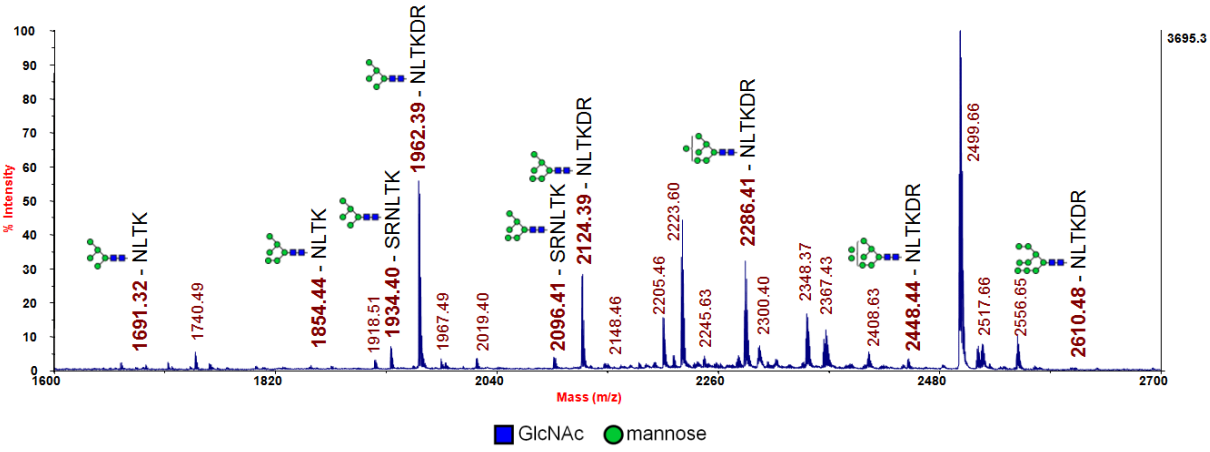


Figure 47: Zoomed MALDI-TOF MS spectra of the bound fraction from ConA TopTip RNase B tryptic glycopeptides enrichment. Identified glycopeptides are highlighted in bold and their structures are shown in the spectrum. DHB was used as a matrix.

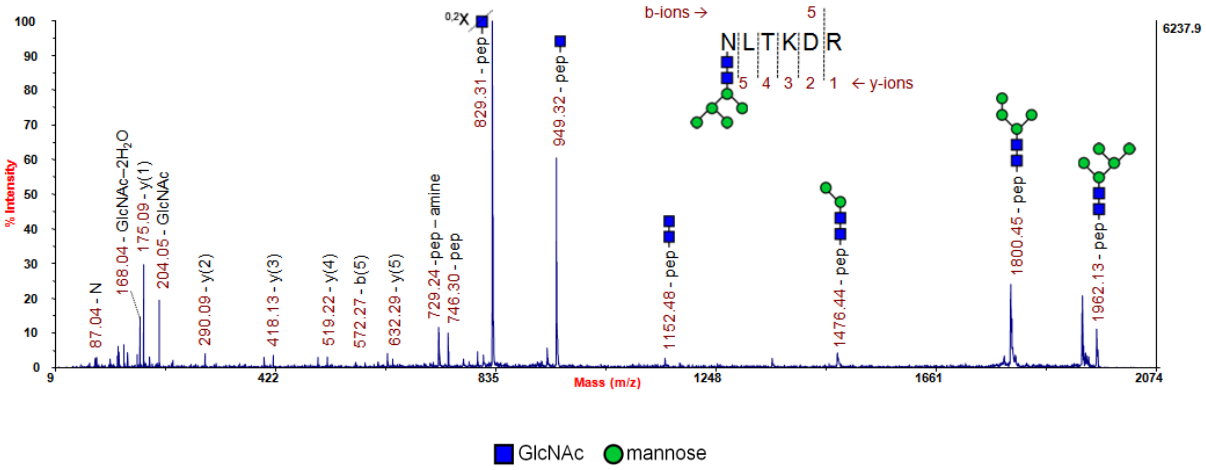


Figure 48: MALDI-TOF/TOF MS/MS fragmentation spectrum of the tryptic glycopeptide N_{60}-R_{65} containing $GlcNAc_2Man_5$ N-glycan from RNase B (precursor at m/z 1962.4). DHB was used as a matrix.

The example of MS/MS fragmentation spectrum of tryptic RNase B glycopeptide is shown in Figure 48. This 1962 Da glycopeptide corresponds to peptide N_{60}–R_{65} with attached *N*-glycan composed of two GlcNAc units and five mannose units. Two GlcNAc oxonium ions identified in this spectrum are the markers of glycopeptide fragmentation. All y- fragment ions and also one b-ion resulted from the fragmentation of the peptide moiety were identified in the spectra. Furthermore, specific fragmentation pattern of non-core-fucosylated glycopeptides and the fragments of the glycan moiety were detected. In detail, the characteristic peak doublet with a mass difference of 17 Da is formed by the signal of peptide fragment and peptide fragment that arises from the cleavage of the side-chain amide bond of the glycosylated asparagine. The fragmentation of the glycan moiety was characterized by cleavage of glycosidic bonds as well as a ^{0,2}X-ring fragmentation of the innermost GlcNAc of the chitobiose core.

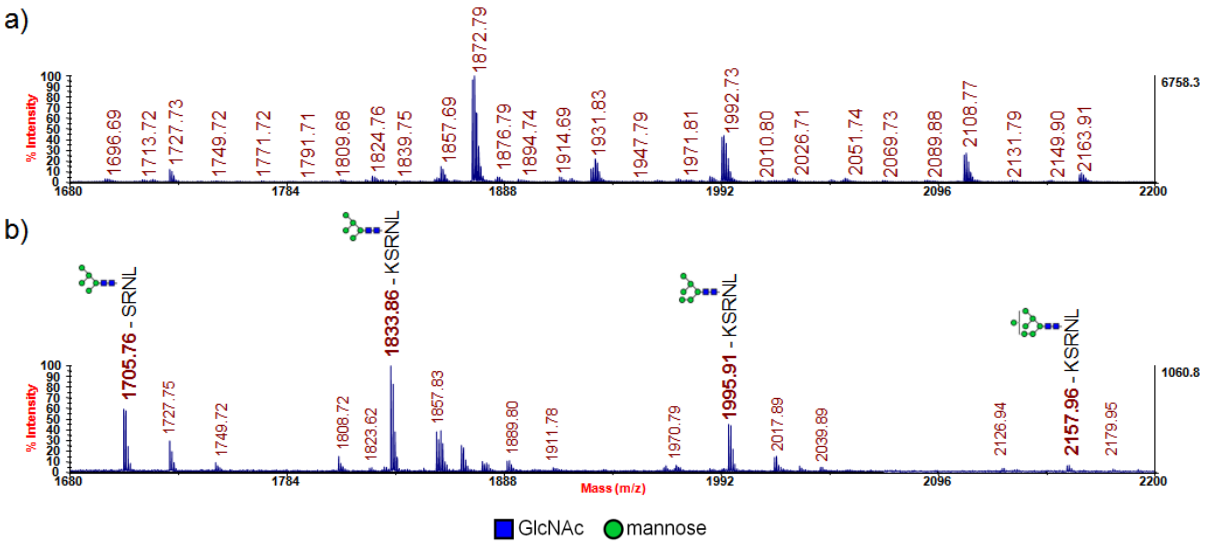


Figure 49: Zoomed MALDI-TOF MS spectra of the a) unbound; and b) bound fraction from ConA TopTip RNase B chymotryptic glycopeptides enrichment. Identified glycopeptides are highlighted in bold and their structures are shown in the spectrum.

RNase B was digested with chymotrypsin as well. The zoomed MS spectrum of the ConA unbound and bound fractions is shown in Figure 49. Significant differences between these two samples were observed. In the bound fraction, several glycopeptides were identified and the intensity of glycopeptide peaks was higher than the intensity of remaining non-glycosylated peptide peaks. In contrast with tryptic digest, glycopeptides containing only one chymotryptic peptide were detected, namely the peptide K_{57}-L_{61} (617 Da) and corresponding glycopeptides with five to eight mannose units. In addition, also one glycopeptide containing non-specifically cleaved peptide S_{58}-L_{61} was identified. This peptide was created by hydrolysis of peptide bond at the C-termini of Lys (K-SRNL), which may be caused by contamination of chymotrypsin by trypsin. According to chymotrypsin manufacturer, less than 0.07 % of trypsin may be present in chymotrypsin.

The Figure 50 shows the MS/MS fragmentation spectrum of chymotryptic RNase B glycopeptide of 1833.9 Da corresponding to peptide K_{57}-L_{61} with attached *N*-glycan of $GlcNAc_2Man_{5-9}$. All identified fragment ions are labelled in the spectrum.

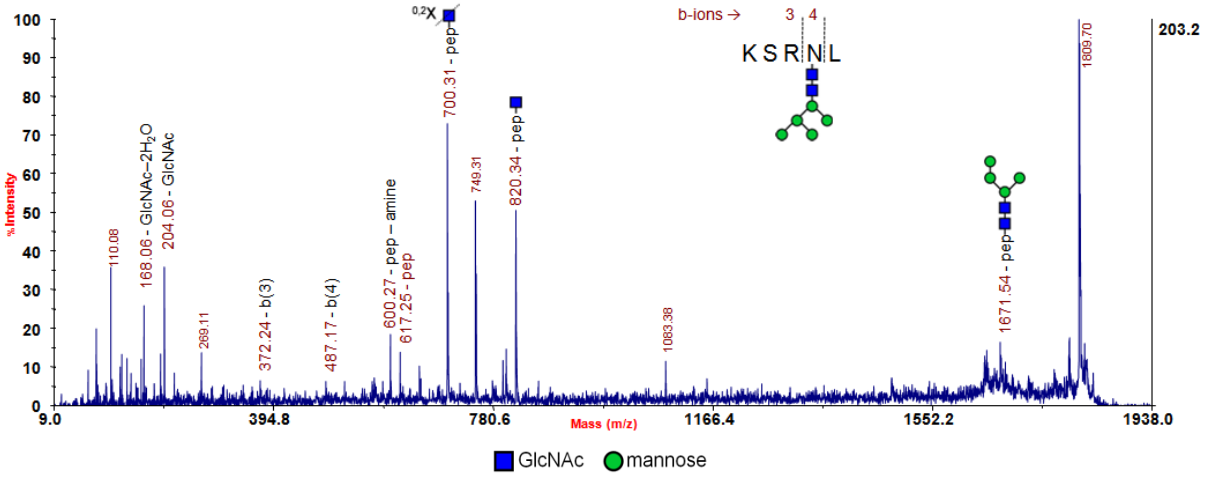


Figure 50: MALDI-TOF/TOF MS/MS fragmentation spectrum of the chymotryptic glycopeptide K_{57}-L_{61} containing $GlcNAc_2Man_5$ N-glycan from RNase B (precursor at m/z 1833.9). DHB was used as a matrix.

In the case of HRP, the identification of glycopeptides was more complicated. While RNase B contains only high-mannose glycans attached to one *N*-glycosylation site, HRP contains nine possible *N*-glycosylation sites and various complex glycans. Wuhrer et al.[115] published that the predominant species at all *N*-glycosylation sites of HRP is xylosylated, core-(α1-3)-fucosylated trimannosyl *N*-glycan structure ($GlcNAc_2Fuc_1Man_3Xyl_1$). Moreover, they have revealed a unusual *N*-glycan structure of Fuc(α1-3)GlcNAc that might arise from *N*-glycan processing by the chitobiose core endoglycosidase cleaving of core-(α1-3)-fucosylated HRP *N*-glycans.[115]

The ConA enrichment of HRP glycopeptides was successful and several glycopeptides were identified. The CID fragmentation of many observed glycopeptides indicated the attached *N*-glycan moiety composition of $GlcNAc_2Fuc_1Man_3Xyl_1$. The examples MS/MS spectra of chymotryptic and tryptic glycopeptides are shown in Figures 51 and 52, respectively. The most prominent signals from the characteristic fragment pattern for core-fucosylated *N*-glycopeptides belonged to peptide after cleavage of side-chain amide bond of the glycosylated asparagine, and to peptide with attached fucosylated GlcNAc.

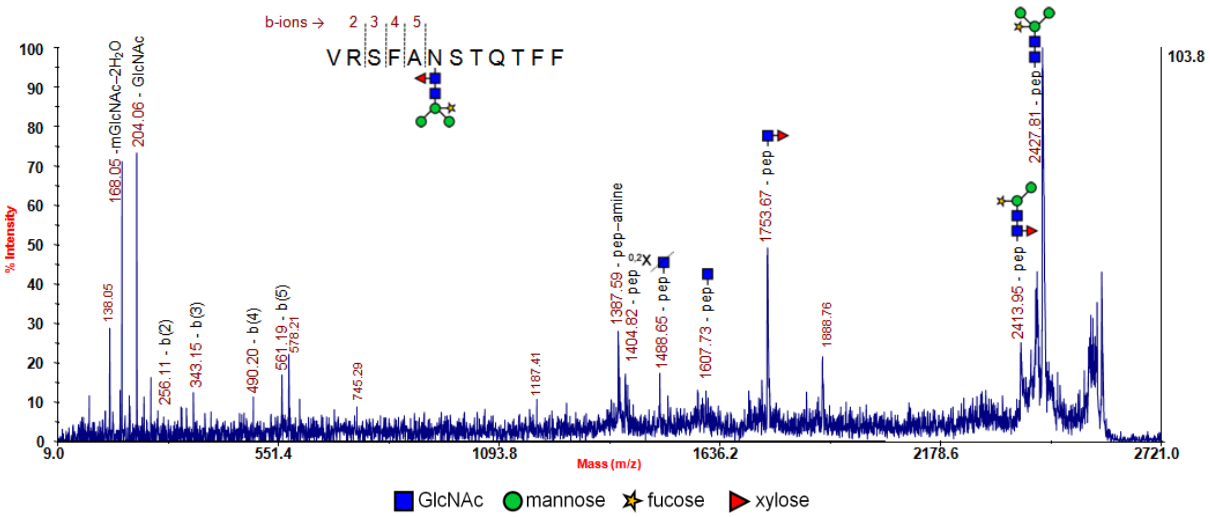


Figure 51: MALDI-TOF/TOF MS/MS fragmentation spectrum of the chymotryptic glycopeptide V_{293}-F_{304} containing $GlcNAc_2Fuc_1Man_3Xyl_1$ N-glycan from horseradish peroxidase (precursor at m/z 2575.3). DHB was used as a matrix.

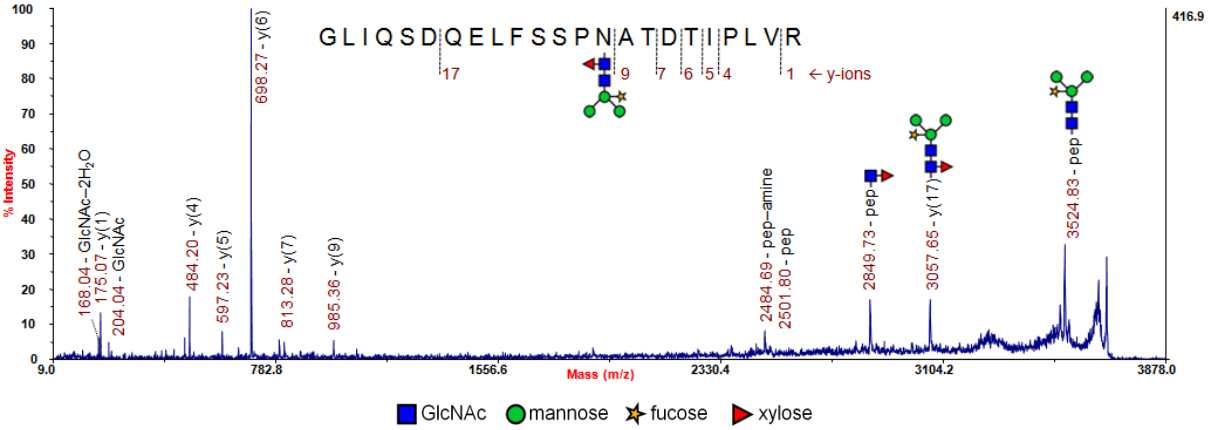


Figure 52: MALDI-TOF/TOF MS/MS fragmentation spectrum of the tryptic glycopeptide G_{272}-R_{294} containing $GlcNAc_2Fuc_1Man_3Xyl_1$ N-glycan from horseradish peroxidase (precursor at m/z 3670.9). Ferulic acid was used as a matrix.

In this study, the enrichment of glycopeptides, subsequent glycopeptide purification and mass spectrometric analysis were successfully optimized. It was found, how certain types of glycopeptides fragment by CID in MALDI-TOF/TOF MS/MS analysis. Moreover, the knowledge about manual evaluation of glycopeptide mass spectra were acquired. Optimized methods and acquired experiences were utilized in subsequent investigation of barley grain and malt glycopeptides.

5.6.4.3. MALDI-TOF MS analysis of barley glycopeptides

After the glycopeptide ConA TopTip enrichment optimization using standard glycoproteins, this method was applied on barley grain and malt water-soluble proteins. First, the glycoprotein fraction was enriched from the aqueous extract of barley grain using the ConA HPLC column. Obtained glycoprotein sample obtained from a total of seven collections was dialyzed against deionized water and freeze dried. Subsequently, purified glycoproteins were digested with chymotrypsin and used for glycopeptides enrichment.

Although chymotrypsin is less specific than trypsin, and moreover, during study of glycopeptides standards also its minor tryptic activity was found, this enzyme was chosen as more suitable for the study of barley glycopeptides due to the assumption of detection more glycopeptides in optimum mass range of MALDI-TOF MS analysis. When some potential glycoproteins identified in barley grain or malt samples are theoretically digested with trypsin[107], only a few or no peptides containing the potential *N*-glycosylation site and smaller than 3 kDa are created.

After carbon purification, MALDI-TOF MS analysis of both bound and unbound ConA TopTip fractions was performed. CHCA, DHB and FA were used as MALDI matrices. Unfortunately, no glycopeptides were detected in the bound fraction. The cause was probably a small concentration of glycoproteins in the sample. In the MS spectrum, only the MS peaks ladder differencing of 162 Da was detected. This ladder indicated the presence of some oligosaccharides that probably originates from the ConA washing buffer. When some glycopeptides were present in the other samples, the oligosaccharide ladder was not detected.

Consequently for glycopeptide quantity increase, the entire aqueous extracts of grain and malt were used for enrichment of glycopeptides. One sample contained 0.5 mg of grain or malt extract. Samples were digested with chymotrypsin and the ConA TopTip glycopeptides enrichment and subsequent carbon purification was performed. Both bound and unbound fractions were analyzed by MALDI-TOF MS. When the MS spectra of the bound and unbound fractions were compared, the intensity decreases or disappearances of some signals, and vice versa, the appearances of some signals were observed in the spectrum of the ConA bound fraction. The example of comparison of the unbound and bound fraction of barley malt including the indication of possible glycopeptides is shown in Figure 53.

After MS/MS fragmentation analysis of the bound fraction, several possible glycopeptides containing the glycopeptide markers in the MS/MS spectrum (GlcNAc oxonium ions of *m/z* 168, *m/z* 204 and in some cases also ion of *m/z* 186) were found. Altogether, six potential glycopeptides were found in the grain sample and nine in the malt sample. Nevertheless, the fragmentation was not optimal in all cases and the characteristic glycopeptides fragment pattern was not detected in all spectra. In addition, obtained glycopeptides could come from a wide range of glycoproteins that are present in the complex grain or malt aqueous extract. Therefore, the identification of both peptide and glycan parts of obtained potential glycopeptides was very difficult.

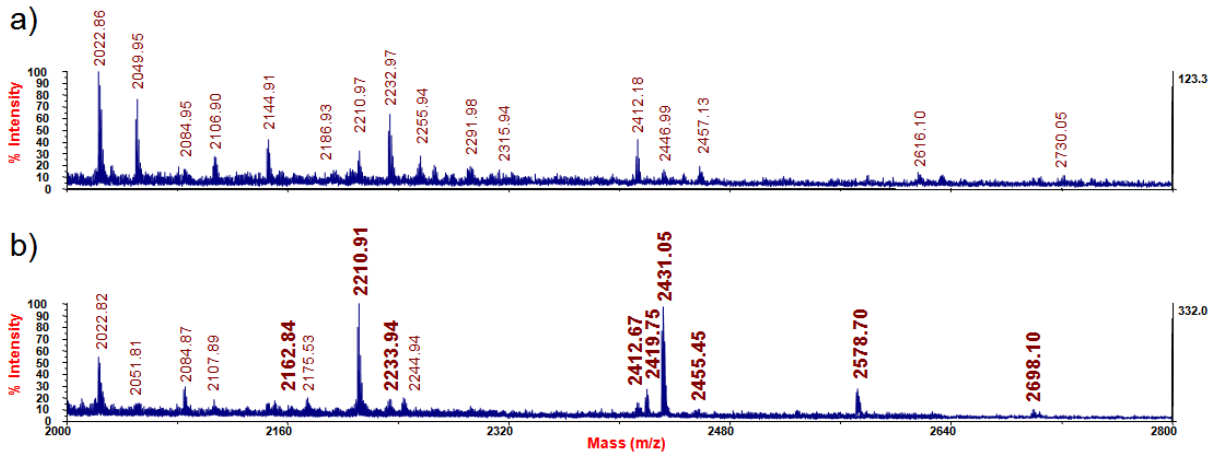


Figure 53: Zoomed MALDI-TOF MS spectra of the a) unbound; and b) bound fraction from ConA TopTip enrichment of barley malt chymotryptic glycopeptides. Identified potential glycopeptides are highlighted in bold. CHCA matrix was used in the case of ConA unbound peptides and DHB matrix for analysis of ConA bound glycopeptides.

The Figures 54 and 55 show the MALDI-TOF/TOF MS/MS spectra of barley grain glycopeptide of *m/z* 2877 and *m/z* 2925, respectively. Identified fragments are listed in the spectra. According to finding of characteristic GlcNAc fragmentation pattern ions in the MS spectra it was deduced that both these glycopeptides probably contained high-mannose glycans $GlcNAc_2Man_6$. Consequently, the masses of peptide moiety were approximately 1498 Da (from glycopeptide of 2877 Da) and 1546 Da (from glycopeptide of 2925 Da). These peptides differ of about 48 Da, which may represent the oxidation of Cys residue. However, the y- and b- fragments of the peptide backbone detected in the spectra showed no similarity, which suggested a different origin of these glycopeptides. These values were manually compared with masses of peptides (containing potential *N*-glycosylated Asn) that were obtained after theoretical chymotrypsin cleavage[107] of proteins identified after ConA HPLC enrichment in our previous study (chapters 5.6.1 and 5.6.2). Moreover, modified MS/MS data were also submitted to the Mascot database searching. Nevertheless, the peptide moiety was not identified by any of these methods.

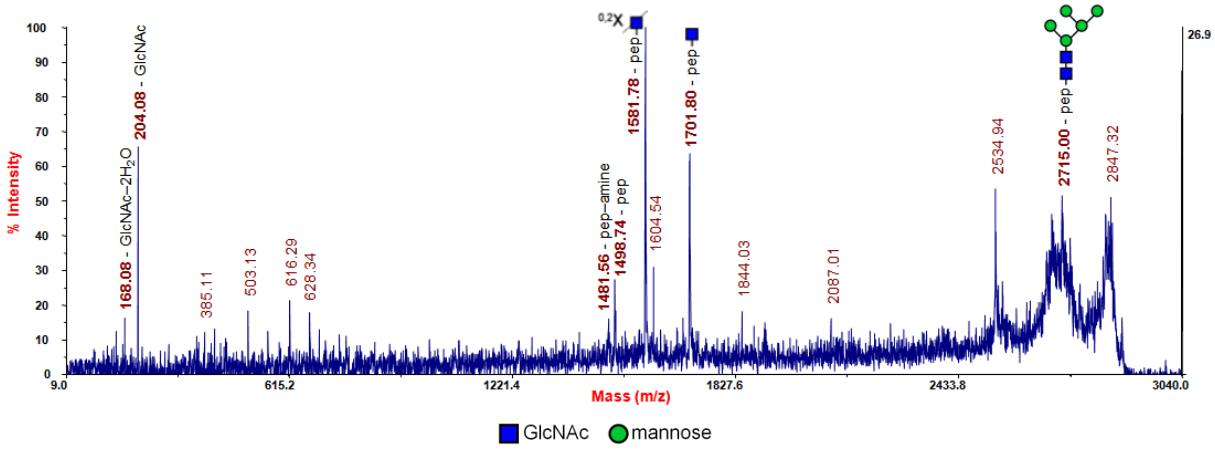


Figure 54: MALDI-TOF/TOF MS/MS fragmentation spectrum of the chymotryptic glycopeptide from barley malt (precursor at m/z 2877). DHB was used as a matrix.

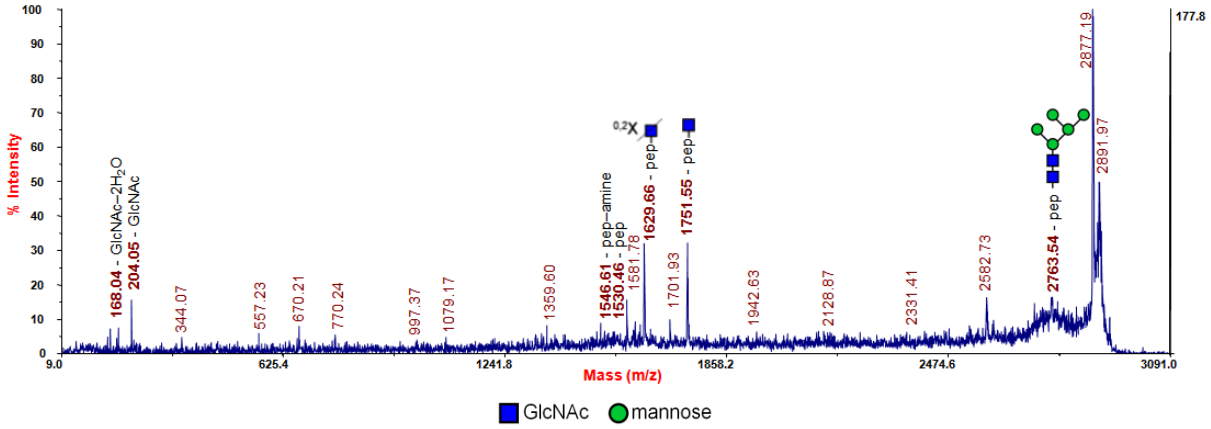


Figure 55: MALDI-TOF/TOF MS/MS fragmentation spectrum of the chymotryptic glycopeptide from barley malt (precursor at m/z 2925). DHB was used as a matrix.

The glycopeptides enrichment method was successfully applied to both standard and real barley samples. However, for improvement of glycopeptide analysis and for facilitation of their identification, the separation of proteins before the proteolytic digestion and glycopeptides enrichment seemed to be necessary. The purification of one or a small group of proteins would be optimal.

6. CONCLUSIONS

The barley grain proteins are significantly changed during the malting and brewing process. The aim of this doctoral thesis was to contribute to the understanding of these protein changes and to perform various proteomic studies of barley grain and malt proteins. A special attention was paid to post-translational modifications of barley proteins, namely non-enzymatically forming glycations and enzymatically forming *N*-glycosylations of barley proteins.

Barley water-soluble proteins in the individual steps of the malting and brewing process were successfully identified. During the barley germination, several proteins are formed. For example α-amylase, β-D-xylosidase, 26 kDa endochitinase 1 or chitinase, were identified in the barley malt sample and were not detected in the grain sample. Therefore, either undetectable amount is present in the barley grain, or these proteins are starting to create during malting. The first signs of α-amylase appeared on Coomassie stained 1D gel from the second day of malting. Moreover, the amount of some proteins seemed to decrease as well, for example the α-amylase/subtilisin inhibitors. Moreover, chymotrypsin inhibitors were detected in the grain sample only.

During mashing, the protein amount is significantly decreasing. In the sweet wort sample α-amylase, β-D-xylosidase, barperm and thaumatin-like proteins were still identified; however, these proteins were precipitated during brewing and were not detected in the wort and green beer samples. In the wort and green beer sample, only protein Z and low-molecular weight proteins (protease/α-amylase inhibitors and ns-LTP) were identified.

To achieve mentioned protein identification, the separation of the complex protein mixture was necessary. Several separation techniques were successfully used, including 1D and 2D gel electrophoresis and reversed phase C18 and SEC HPLC.

SDS-PAGE was primarily used for the separation of barley proteins and together with subsequent in-gel protease digestion and MALDI-TOF MS analysis represented the most appropriate technique for the rapid and efficient identification of barley proteins. Moreover, it was a suitable method for successful monitoring of changes in the protein profile during individual steps of the malting and brewing process.

Two-dimensional electrophoresis allowed more effective separation of individual proteins present in barley grain as well as the approximate determination of the isoelectric points of barley proteins. Some additional proteins were identified in comparison with 1D GE (SDS-PAGE).

The reversed phase C18 HPLC separation represents a suitable method for rapid barley protein separation and monitoring of protein profile changes during malting and brewing. Because of the rapidity and simplicity of this method, it was used also for the comparison of individual barley varieties. This method also showed good long-term reproducibility. The comparison of C18 HPLC grain or malt profiles could possibly be used for barley varieties discrimination.

SEC HPLC separation was successfully used for separation and analysis of barley and malt proteins. The differences between grain and malt protein profiles were studied as well. The peak corresponding to β-amylase, β-glucosidase and protein Z showed significant increase during malting, and therefore, this fraction was used for detailed relative quantification analysis using the iTRAQ method. It was found that the amount of β-amylase increased the most in comparison to the two others proteins. If the malt protein content

is expressed as 100 %, approximately 60 % of malt β-amylase is present in grain sample. Accordingly, approximately 80 % of malt β-glucosidase and 83 % of malt protein Z are present in grain.

The low-molecular weight proteins and their changes during malting and mashing were successfully analyzed using the linear mode of MALDI-TOF MS. Between the greatest advantages of this method belong its rapidity as well as the rapid estimation of the level of glycations of barley low-molecular weight proteins. C-terminal 363 – 399 fragment of protein Z, ns-LTP1, ns-LTP1b (LTP1 with bound lipid-like molecule) and ns-LTP2 were among studied proteins. Protein Z fragment was formed during malting and resulted in the most intensive peak of the malt MS spectrum. LTP2 and LTP1b were detected in all three investigated samples of grain, malt and sweet wort. LTP1 was not detected in the grain and malt samples; however, it was more intensive than LTP1b in the sweet wort sample. It follows that the lipid-protein bond was breaking up during mashing.

All mentioned low-molecular weight proteins were non-enzymatically glycated during the malting process. The degree of glycation within individual steps of malting was successfully monitored. LTP1b and LTP2 glycated forms were detected from the 3rd day of the malting and these proteins were gradually glycated with up to three and two hexose units, respectively. Protein Z fragment was detected from the 3rd day of the malting process and its glycation with one hexose unit was slightly detected from the 4th day of the malting process. The glycation of protein Z was analyzed by MALDI-TOF MS after SDS-PAGE separation and chymotrypsin digestion as well. The glycation of Lys_{276} residue by one hexose unit was found.

In addition to water-soluble proteins, barley prolamins (hordeins) were studied as well. SDS-PAGE separation of alcohol-soluble extracts allowed successful identification of hordeins and the monitoring of their changes during the whole malting process. The content of hordeins was slightly decreased during malting and the most significant decrease was observed in the case of D hordein. For obtaining of more detailed view on the changes of C hordein during malting, relative quantification using iTRAQ method was performed. It was found that the amount of C hordein was significantly decreased and the amount of C hordein in malt represented 35 % of the initial amount in barley grain.

Moreover, the next part of the thesis was focused on study of enzymatically formed *N*-glycosylations of water-soluble proteins. ConA affinity chromatography was used for glycoprotein enrichment, and subsequently, captured proteins were separated by SDS-PAGE and analyzed by MALDI-TOF MS. Two ConA columns were used, a manually filled column with ConA-agarose and the monolithic ConA HPLC column. Using both types of columns, several potential glycoproteins were identified in barley grain as well as malt. Nevertheless, ConA was bleeding from the manually filled column, which made the analysis more difficult. Therefore, it was replaced by monolithic HPLC column with firmly bound stationary phase in the next studies. Thereby, faster, semi-automated and more reproducible separation was achieved. Certain proteins identified in the ConA bound fractions are also proven glycoproteins according to the database. However, several proteins, especially in barley grain, are still named "predicted proteins". This implies that this area of barley minor glycoproteins is still little studied and these studies are still a current issue.

In the last part of the thesis, the optimization of the oligosaccharide moiety of glycoproteins was performed. By several studies it was found that the analysis of the glycan part using polyacrylamide gel separated proteins is complicated. Glycans/glycopeptides are probably tightly crosslinked in the gel and were not extracted from the gel. Therefore, the sample had to remain in the solution. The glycan part of standard glycoproteins was studied by two ways: by analysis of glycan after protein deglycosylation, and by analysis of glycopeptides after their enrichment. The second approach seemed to be more promising for barley proteins, because allows the simultaneous analysis of both the peptide and glycan part of glycoproteins.

After ConA glycopeptides enrichment of barley grain and malt chymotryptic digests, some glycopeptides were detected. Nevertheless, their identification was very difficult. For improvement of glycopeptide analysis and for facilitation of their identification, the separation of proteins before the proteolytic digestion seemed to be necessary.

Used method of glycopeptides enrichment will be applied in further studies of glycosylated proteins in various cereals and cereal food products focused on glycoproteins associated with allergenicity. Moreover, the glycopeptide or glycoprotein enrichment using more types of lectin or further optimizations of deglycosylations and glycan labelling for the improvement of MS spectra could be performed in future studies.

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8. ABBREVIATIONS

AC	affinity chromatography
ACN	acetonitrile
AGE	advanced glycosylation end product
BASI	bifunctional α-amylase/subtilisin inhibitor
BDAI	barley dimeric α-amylase/trypsin inhibitor
CHCA	α-cyano-4-hydroxycinnamic acid
CID	collision induced dissociation
ConA	concanavalin A
Da	Daltons
DAC	diammonium citrate
DHAP	2,6-dihydroxyacetophenone
DHB	2,5-dihydoxybenzoic acid
DTT	dithiothreitol
Endo H	endoglycosidase H
ESI	electrospray ionization
ER	endoplasmic reticulum
FA	ferulic acid
GE	gel electrophoresis
Glc	glucose
GlcNAc	N-acetyl-D-glucosamine
GPI	glycosylphosphatidylinositol
HABA	2-(4-hydroxyphenyl)azobenzoic acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
ICAT™	isotope coded affinity tags
IEF	isoelectric focusing
IMAC	immobilized metal-affinity chromatography
IPG	immobilized pH gradient
iTRAQ	isobaric tags for relative and absolute quantification
LC	liquid chromatography
MALDI	matrix-assisted laser desorption/ionization
Man	mannose
ME	mercaptoethanol
MS	mass spectrometry
ns-LTP	non-specific lipid transfer protein
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
PI	protease inhibitor
PMF	peptide mass fingerprinting
PNGase	peptide-*N*-glycosidase
PTM	post-translational modification
RIBM	Czech Research Institute of Brewing and Malting, PLC
RNase B	ribonuclease B
PR	pathogenesis related protein

RP	reversed phase
SA	sinapinic acid
SDS	sodium dodecylsulfate
SEC	size exclusion chromatography
SILAC	stable isotope labelling with amino acids in cell culture
TEMED	tetramethylethylenediamine
TFA	trifluoroacetic acid
THAP	2,4,6-trihydroxyacetophenone
TOF	time-of-flight
UV	ultraviolet

9. SUMMARY OF APPENDICES

10.1 Barley proteins identified after tryptic digestion

10.2 Barley proteins identified after chymotryptic digestion

10.3 Curriculum vitae

10.4 List of publications

10.5 Conference participations

10. APPENDIX

10.1. Barley proteins identified after tryptic digestion

The summary of barley proteins identified after tryptic digestion in the entire doctoral thesis, including corresponding identified peptides and their sequences.

protein name	NCBInr entry	UniProtKB entry	mass [Da]	$[M + H]^+$ (observed)	peptide sequence
1,3-beta-glucan endohydrolase GII	gi|809429	P15737	32.4	1424.76	IYFADGQALSALR
				1618.84	IGVCYGVIGNNLPSR
				2077.01	VVVSESGWPSAGGFAASAGNAR
				2138.06	DNPGSISLNYATFQPGTTVR
				2147.10	YIAAGNEVQGGATQSILPAMR
				2200.19	LLASTGAPLLANVYPYFAYR
				2355.21	VSTSIRFDEVANSFPPSAGVFK
26 kDa endochitinase 1	gi|2506281	P11955	34.4	1743.86	GPIQLSHNYNYGPAGR
				1756.83	GFYTYDAFVAAASAFR
chain A, the refined crystal structure of an endochitinase 26 kDa endochitinase 2	gi|157834680 gi|116316	P23951	28.2	1743.84	GPIQLSHNYNYGPAGR
				2039.86	GASSDYCTPSAQWPCAPGK
				2195.95	GASSDYCTPSAQWPCAPGKR
				2497.21	VPGFGVITNIINGGIECGHGQDSR
				2582.12	YCDILGVGYGNNLDCYSQRPFA
				2958.39	GFYTYDAFVAAAAAFPGFGTTGSADAQKR
aldose reductase	gi|113595	P23901	35.8	981.51	TAITEAGYR
				1259.63	IWCTNLAPER
				1439.75	SGHAMPAVGLGTWR
				1455.74	SGHAMPAVGLGTWR + Oxidation (M)
				1682.88	HGIHVTAYSPLGSSEK
				1959.99	VLTGEELFVNKTHGPYR
				2244.59	DLQLDYIDLYHIHWPFR
alpha-amylase	gi|166985 gi|229610883	Q03651 C3W8M9	47.7	1052.48	TDVGFDGWR
				1121.64	AIADIVINHR
				1284.60	LDWGPHMICR
				1455.79	GILNVAVEGELWR
				1555.72	GIYCIFEGGTPDAR
				2645.22	AVTFVDNHDTGSTQHMWPFPSDR
alpha-amylase 1	gi|166979	Q40016	48.2	1052.48	TDVGFDGWR
				1121.64	AIADIVINHR
				1455.79	GILNVAVEGELWR
				1555.72	GIYCIFEGGTPDAR
				3136.60	VDDIAAAGITHVWLPPASQSVAEQGYMPGR
alpha-amylase inhibitor BDAI-1	gi|123970	P13691	16.4	1941.07	DCCQEVANISNEWCR
				2122.05	SVYAALGVGGGPEEVFPGCQK
				2175.52	LLVAGVPALCNVPIPNEAAGTR
				2232.24	LLVAGVPALCNVPIPNEAAGTR
alpha-amylase inhibitor BMAI-1	gi|2506771	P16968	16.4	1206.62	ATVAEVFPGCR
				2081.92	SQCAGGQVVESIQKDCCR
				2107.98	QIAAIGDEWCICGALGSMR
				2218.11	ELGVALADDKATVAEVFPGCR
alpha-amylase type B isozyme chain A, Amy2BASI PROTEIN-protein complex alpha-amylase	gi|2851583 gi|4699831 gi|229610885	P04063 P04063 C3W8N0	47.5	1036.49	ADIGFDGWR
				1121.64	AIADIVINHR
				1284.60	LDWGPHMICR
				1455.79	GILNVAVEGELWR
				1555.72	GIYCIFEGGTPDAR
				2645.22	AVTFVDNHDTGSTQHMWPFPSDR
				3136.60	VDDIAAAGITHVWLPPASQSVAEQGYMPGR
				3287.65	SEPSFAVAEIWTSLAYGGDGKPNLNQDQHR
alpha-amylase/trypsin inhibitor CMa	gi|585289	P28041	15.5	1068.54	SHPDWSVLK
				1296.67	DLPGCPKEPQR
				1702.68	CCQELDEAPQHCR
alpha-amylase/trypsin inhibitor CMb	gi|585290	P32936	16.5	1023.50	EVQMDFVR
				1167.65	DYVEQQACR
				1799.87	SRPDQSGLMELPGCPR
				1815.86	SRPDQSGLMELPGCPR + Oxidation (M)
				1861.82	QQCCGELANIPQQCR

protein name	NCBInr entry	UniProtKB entry	mass [Da]	$[M + H]^+$ (observed)	peptide sequence
alpha-amylase/trypsin inhibitor CMd CMd preprotein (AA -14 to 146)	gi|585291 gi|758343	P11643	18.5	1813.86	DYVLQQTCAVFTPGSK
				1876.01	LLVAPGQCNLATIHNVR
				1967.87	LYCCQELAEIPQQCR
				3387.66	YFMALPVPSQPVDPSTGNVGQSGLMDLPGCPR
				3403.68	YFMALPVPSQPVDPSTGNVGQSGLMDLPGCPR + Oxidation (M)
barwin chain A, Three-Dimensional Structure In Solution Of Barwin	gi|114832 gi|159162134	P28814	13.7	1299.71	VTNPATGAQITAR
				1439.66	YGWTAFCGPAGPR
				1654.79	SKYGWTAFCGPAGPR
				2267.07	IVDQCANGGLDLDWDTVFTK
				2826.31	IDTNGIGYQQGHLNVNYQFVDCRD
basic pathogenesis-related protein PR5	gi|2344818	O23997	25.2	1057.44	TGCTFDGSGR
Barperm1	gi|2454602	O22462		1135.43	FGGDTYCCR
thaumatin-like protein TLP6	gi|14164979	Q946Z0		1913.35	LDPGQSWALNMPAGTAGAR
thaumatin-like protein TLP7	gi|14164981	Q946Y9		2062.70	VSGQQPTTLAEYTLGQGANK
beta-amylase	gi|10953877	Q9FUK6	59.6	838.45	MHANLPR
				1016.58	LFGFTYLR
				1189.63	ISGIHWWYK
				1299.58	ASINFTCAEMR
				1326.67	YDPTAYNTILR
				1370.68	DPYVDPMAPLPR
				1386.69	DPYVDPMAPLPR + Oxidation (M)
				1442.71	EGLNVACENALPR
				1515.81	FFLAWYSNNLIK
				1669.74	SAVQMYADYMTSFR
				1685.71	SAVQMYADYMTSFR + Oxidation (M)
				1724.84	LSNQLVEGQNYANFK
				1738.85	NARPHGINQSGPPEHK
				1811.93	SGPEISIEMILQAAQPK
				2013.96	VPSHAAELTAGYYNLHDR
				2025.84	DVGTCDPDIFYTDGHGTR
				2086.05	NIEYLTLGVDNQPLFHGR
				2190.09	MHANLPRDPYVDPMAPLPR
				2251.17	GNYVQVYVMLPLDAVSVNNR
				2733.26	DSEQSSQAMSAPEELVQQVLSAGWR
				2749.34	DSEQSSQAMSAPEELVQQVLSAGWR + Oxidation (M)
				2841.28	AAAAAVGHPEWEFPNDVGQYNDTPER
				3007.54	LQAIMSFHQCGGNVGDAVNIPIPQWVR
beta-D-xylosidase	gi|18025342	Q8W011	84.4	825.51	YAAVFVR
				960.51	LGLFDGNPK
				1003.54	LPVTWYPK
				1043.60	IGQVIGTEAR
				1251.63	EHQDLALQAAR
				1423.68	HFTAYDLENWK
				2467.25	GVYNNGQAEGLTFWAPNINVFR
				2490.32	AATSFPQVILTAASFNPHLWYR
				3062.65	IGAIVWAGYPGQAGGIAIAQVLFGDHNPGGR
beta-glucosidase	gi|804656	Q40025	57.7	882.46	FSISWSR
				973.45	NMGFDAYR
				1078.54	LPGFSADESR
				1210.59	NWFTFNEPR
				1356.62	VNQEGVDYYNR
				1437.74	VKNWFTFNEPR
				1571.86	FGIVYVDFNTLKR
				1617.83	IVGNRLPGFSADESR
				1637.78	IVGAFADYAEFCFK
				1643.83	VVAALGYDNGFHAPGR
				2172.00	IFPDGTGKVNQEGVDYYNR
				2233.20	TEPYIVTHNIILSHAAAVQR
				2362.11	QGFPAGFVFGTAASAYQVEGMAR
beta-glucosidase [Sofia, Peptide Partial, 41 aa, segment 1 of 6]	gi|544867	not mapped	4.2	1694.79	DGPNPNPEIGNTGGLSR
				2362.11	QGFPAGFVFGTAASAYQVEGMAR
bifunctional alpha-amylase/subtilisin inhibitor	gi|18916	F2E8J4	22.5	1182.53	YSGAEVHEYK
				1318.64	AHGGGLTMAPGHGR
				1334.63	AHGGGLTMAPGHGR + Oxidation (M)
alpha-amylase/subtilisin inhibitor	gi|123974	not mapped		1356.64	ADANYYVLSANR
amylase subtilisin inhibitor alpha chain C, Amy2BASI PROTEIN-protein complex from barley seed	gi|225172	not mapped		1529.86	ITPYGVAPSDKIIR
				1546.83	HVITGPVKDPSPSGR
	gi|4699833	P07596		1843.77	LMSCGDWCQDLGVFR
				2307.03	HCPLFVSQDPNGQHDGFPVR
				2409.07	AYTTCLQSTEWHIDSELAAGR
BTI-CMe2.1	gi|6634471	P01086	16.8	1544.77	TYVVSQICHQGPR

protein name	NCBInr entry	UniProtKB entry	mass [Da]	$[M + H]^+$ (observed)	peptide sequence
CMd3 protein	gi|2264392	O24000	18.5	1876.03	LLVAPGQCNLATIHNVR
CMd subunit of tetrameric alpha-amylase inhibitor	gi|2266660	not mapped		1966.87	LYCCQELAKIPQQCR
				3387.70	YFMALPVPSQPVDPSTGNVGQSGLMDLPGCPR
cold-regulated protein	gi|10799810	Q9FSI8	17.6	1544.13	SLALVVQDIDADER
				1714.24	DISPPLEWYGVPGGAR
				1842.35	KDISPPLEWYGVPGGAR
cytosolic glutathione reductase	gi|157362219	A8CCK8	53.1	2152.66	VVTDKGDEFIADVVLFATGR
dehydrin	gi|6017948	Q9ZTR8	23.4	2226.47	VDEYGNPVPPVDQYGNPIPR
endosperm-specific beta-amylase 1	gi|29134857	Q84T19	59.6	1016.56	LFGFTYLR
beta-amylase 1	gi|38349539	Q6SNP7		1326.67	YDPTAYNTILR
beta-amylase	gi|10953875	Q9FUK7		1752.89	LSNQLVEGQNYVNFK
				2086.07	NIEYLTLGVDNQPLFHGR
ent-kaurene synthase-like protein 2	gi|49065964	Q673F8	23.9	1286.70	LQKPIDTCRR
fructose-bisphosphate aldolase	gi|226316443	C1J960	38.7	1338.69	KPWNLSFSFGR
				2069.06	IGATEPSQLSIDQNAQGLAR
				2381.17	GTIELAGTNGETTTQGFDDLGKR
germin B	gi|9837113	Q9FYY4	24.6	1786.07	IDYGPLGVNTPHIHPR
germin F	gi|9837115	Q9FYY3			
germin D	gi|9837117	Q9FYY2			
glucose and ribitol dehydrogenase homolog	gi|7431022	F2CSK4	31.9	834.51	GAIVAFTR
				1237.64	VVEEVANAHGGR
				1483.64	ALSGDLGYEENCR
glyceraldehyde-3-phosphate dehydrogenase	gi|126467754	A3RHT3	25.1	1498.66	VPTVDVSVVDLTVR
				1662.75	TLLFGEKPVTVFGVR
glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	gi|120680	P26517	36.5	1142.54	YDTVHGHWK
				1498.84	VPTVDVSVVDLTVR
				1662.95	TLLFGEKPVTVFGVR
				1788.79	LVSWYDNEWGYSNR
				2189.01	GIMGYVEEDLVSTDFVGDSR
glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	gi|120668	P08477	33.2	1133.54	YDTVHGQWK
				1498.84	VPTVDVSVVDLTVR
				1775.80	LVSWYDNEWGYSTR
				2186.02	GILGYVDEDLVSTDFQGDSR
grain softness protein	gi|54661662	Q5ITH7	18.3	1840.17	SCEEVQDQCCQQLR
chain C, Amy2BASI PROTEIN-Protein Complex From Barley Seed	gi|4699833	P07596	22.2	1182.53	YSGAEVHEYK
				1318.63	AHGGGLTMAPGHGR
				1334.61	AHGGGLTMAPGHGR + Oxidation (M)
				1356.63	ADANYYVLSANR
				1529.86	ITPYGVAPSDKIIR
amylase subtilisin inhibitor alpha	gi|225172	not mapped		1546.83	HVITGPVKDPSPSGR
thioredoxin H2	gi|119390312	P07596		1655.77	ADPPPVHDTDGHELR
alpha-amylase/subtilisin inhibitor (BASI)	gi|123974			1843.78	LMSCGDWCQDLGVFR
				1859.78	LMSCGDWCQDLGVFR + Oxidation (M)
				2307.05	HCPLFVSQDPNGQHDGFPVR
				2409.08	AYTTCLQSTEWHIDSELAAGR
				2565.17	AYTTCLQSTEWHIDSELAAGRR
				2993.39	ADPPPVHDTDGHELRADANYYVLSANR
chitinase	gi|563489	Q43765	26.6	1011.41	ATSPPYYGR
chitinase II	gi|9501334	Q9LEH7		1689.86	GPIQLTGQSNYDLAGR
	gi|215512228	D2CVR3		1909.92	ELAAFFGQTSHETTGGTR
late embryogenesis abundant protein B19.1A	gi|547817	Q05190	10.0	1315.93	SLEAQQNLAEGR
				1501.92	EQMGQEGYSEMGR
low-molecular-weight glutenin subunit group 3 type II [Triticum aestivum]	gi|17425184	Q8W3W6	27.2	1568.63	TTTRVPFGVGTGVGGY
				1677.66	TLPTMCNVNVPLYR
				1693.84	TLPTMCNVNVPLYR + Oxidation (M)
				2059.80	VFLQQQCSPVAMPQSLAR
				2076.04	VFLQQQCSPVAMPQSLAR + Oxidation (M)
LTP 1	gi|19039	P07597	12.3	1327.60	DLHNQAQSSGDR
Chain A, Non-Specific Lipid Transfer Protein 1	gi|47168353			1662.87	GIHNLNLNNAASIPSK
				2009.86	CNVNVPYTISPDIDCSR
Non-specific lipid-transfer protein 1	gi|128376			2331.02	DLHNQAQSSGDRQTVCNCLK
Lipid Transfer Protein Complexed With Palmitate	gi|157830246				
	gi|326533572	F2ED95		2524.08	MKPCLTYVQGGPGPSGECCNGVR
malate dehydrogenase	gi|326490940	F2D4W6	35.9	2000.10	VLVTGAAGQIGYALVPMIAR
				2390.13	ELVQDDEWLNGEFIATVQQR
				2603.27	GVVATTDPVEACTGVNVAVMVGGFPR
pathogenesis-related protein 4	gi|1808651	P93180	15.7	1299.70	VTNPATGAQITAR
pathogenesis-related protein PRB1-2	gi|548588	P35792	17.7	1355.66	GVFITCNYEPR
Pathogenesis-related protein PRB1-3	gi|548589			1404.70	VCGHYTQVVWR
Pathogenesis-related protein 1	gi|548592	Q05968		1423.72	LQAFAQNYANQR

protein name	NCBInr entry	UniProtKB entry	mass [Da]	$[M + H]^+$ (observed)	peptide sequence
peroxidase BP 1 chain A, crystal structure of barley grain peroxidase 1	gi|167081 gi|157830301	Q40069	39.3	1772.90	DSVVVSGGPDYRVPLGR
				1843.98	LFPRPDPTISPTFLSR
				1970.96	TPNVFDNKYYIDLVNR
				2520.28	EGLFVSDQDLFTNAITRPIVER
				2872.44	SFASTQDVLSDLPGPSSNVQSLLALLGR
				2942.41	LGLDATDLVTISGGHTIGLAHCSSFEDR
				1055.55	YYIDLVNR
				1502.78	GAVVSCSDILALAAR
PR-1a pathogenesis related protein (Hv-1a)	gi|401831	Q43489	17.4	1404.70	VCGHYTQVVWR
				1412.71	LQAYAQSYANQR
predicted protein	gi|326490934 gi|326493636	F2D4W3 F2CR08	48.2	978.53	FRAPVEPY
				1790.99	AAVPSGASTGVYEALELR
				1983.92	GNPTVEVDVCCSDGTFAR
				2132.09	QLVLPVPAFNVINGGSHAGNK
				2604.25	MTEECGEQVQIVGDDLLVTNPTR
predicted protein	gi|326491097	F2EE76	11.8	973.53	KPFPHGYK
				1057.50	VGYVANFCK
				1107.57	YQKFPAEPK
				1505.67	LAPSDACCAVWQK
predicted protein	gi|326493416	F2CQP8	32.8	889.48	MLHAVYR
				1278.62	ITSFLDPDGWK
				2397.71	GGSTVIAFAQDPDGYLFELIQR
predicted protein	gi|326522492	F2EK36	34.4	1743.86	GPIQLSHNYNYGPAGR
				1756.83	GFYTYDAFVAAASAFR
				2609.18	YCDILGVGYGNNLDCYNQRPFA
predicted protein	gi|326497617	F2EEX6	36.0	1475.64	VYSVQLQALDALR
				1864.73	DIQLNYATFQPGATAVR
predicted protein	gi|326520285	F2EJ79	15.4	1082.46	GAVPVSAPEQK
				1579.59	SVAFYADAFGYNVR
predicted protein	gi|326520537	F2EJK5	22.9	1478.01	SGAFFFISSDEDR
				2031.36	DGWVVDPAESYNHWAER
predicted protein	gi|326502266	F2DJC5	26.1	1187.84	TVHFWQVDR
				1514.13	GGVLFMPGVPGVVER
predicted protein	gi|326506996	F2DKF4	12.4	2005.15	GPVEICFDYDDVDAAYR
predicted protein	gi|326502776	F2DV95	80.9	1477.96	HVAVASVWGLVALR
				1608.98	VTPYDVLLSYPVSR
				2375.33	DAWTFGAADPNSGTAALLELAQR
predicted protein	gi|326502492	F2DJN8	112.8	1009.54	QLEFFIGR
				1447.73	FIYVEQAFFQR
predicted protein	gi|326513840	F2CYL7	55.8	1213.66	YFPFVQVASR
				1367.75	LVESEGGSVHVVR
				1555.90	NKPQFLVGPTSVLR
				1603.95	GGPFVFFGFTTSALR
				1818.84	APEPYNLFDHEPSFR
				1945.19	VILGPELAAGLGVPLKELR
				2295.06	R.NTYGWSISVDKHDYEPLDR
				2773.22	GVPGSGVPELPWQHGHGGWSAGCGACR
predicted protein (heat shock protein)	gi|326497219	F2E4C2	71.9	1215.64	DAGVIAGLNVMR
				1358.60	NALENYAYNMR
				1434.72	MVNHFVQEFKR
				1487.68	TTPSYVAFTDTER
				1540.74	ARFEELNMDLFR
				1691.70	STAGDTHLGGEDFDNR
				1787.96	IINEPTAAAIAYGLDKK
				1821.89	NQVAMNPTNTVFDAKR + Oxidation (M)
				1869.96	MDKTQVHEIVLVGGSTR
				2658.23	EQVFSTYSDNQPGVLIQVYEGER
protein z-type serpin	gi|1310677	P06293	43.3	925.42	LSIAHQTR
				1137.52	LVLGNALYFK
				1144.47	LRSAISSNPER
				1413.58	ISYQFEASSLLR
				1653.66	LSTEPEFIENHIPK
				1688.71	FKISYQFEASSLLR
				1809.75	RLSTEPEFIENHIPK
				2529.03	ELNALAEQVVQFVLANESSTGGPR
				2622.16	AAGNVAFSPLSLHVALSLITAGAGGATR
protein Z (180 AA)	gi|19079	P06293	43.3	1413.58	ISYQFEASSLLR
				1653.66	LSTEPEFIENHIPK
				1809.75	RLSTEPEFIENHIPK
purple acid phosphatase isoform a	gi|237847799	C4PKL2	60.3	1328.64	STPIHETYQPR
				1812.74	NQDLYGSAGDEIYIVR
putative avenin-like a precursor	gi|326501830	F2EGD5	19.0	1816.91	QQCCQPLAQISEQAR
Rar1	gi|6581046	Q9SE34	25.4	1136.50	QGVETEACSR

protein name	NCBInr entry	UniProtKB entry	mass [Da]	$[M + H]^+$ (observed)	peptide sequence
serine carboxypeptidase II, chain A	gi|20455471	P08818	53.0	1091.60	GAGLVLNEYR
				1784.84	TAHDSYAFLAAWFER
				1784.85	TAHDSYAFLAAWFER
				1784.88	TAHDSYAFLAAWFER
				2630.23	LPGQPEVDFDMYSGYITVDEAAGR
				2651.30	EFYVAGESYAGHYVPELSQLVHR
				2998.50	IVRLPGQPEVDFDMYSGYITVDEAAGR
subtilisin-chymotrypsin inhibitor CI-1A	gi|124125	P16062	8.9	973.51	VFVLVAVAR
				2734.05	DKPNAQVEVIPVDAMVHLNFDPNR + Oxidation (M)
subtilisin-chymotrypsin inhibitor CI-1B	gi|124127	P16063	9.0	1001.53	IFILVAVAR
				2709.00	DKPDAQIEVIPVDAMVPLDFNPNR + Oxidation (M)
subtilisin-chymotrypsin inhibitor-2A chymotrypsin inhibitor-2	gi|124122 gi|158530106	P01053 A8V4D2	9.4	1627.68	LFVDKLDNIAQVPR
thaumatin-like protein TLP8	gi|14164983	Q946Y8	24.3	1269.67	VITPACPNELR
				1769.79	AAGGCNNACTVFKEDR
				1930.97	LDAGQTWSINVPAGTTSGR
tissue-ubiquitous beta-amylase 2	gi|61006818	Q4VM11	56.9	1769.72	SAPEELVQQVLSAGWR
				2086.81	NIEYLTLGVDDQPLFHGR
triosephosphate isomerase	gi|2507469	P34937	26.9	954.46	FFVGGNWK
				1374.69	VIACVGETLEQR
				1381.66	SLLGESSEFVGEK
				1811.94	LRPEIQVAAQNCWVK
trypsin inhibitor CMc	gi|161784337	P34951	15.2	2873.54	AFPPSQSQGAPPQLPPLATECPAEVKR
trypsin inhibitor cme precursor	gi|1405736	P01086	16.1	1544.77	TYVVSQICHQGPR
BTI-CMe1 BTI-CMe3.1 protein Trypsin inhibitor CMe	gi|2707922 gi|2707924 gi|85682780			1614.70	CCDELSAIPAYCR
trypsin/amylase inhibitor pUP13	gi|225102	not mapped	14.7	1205.72	ELSDLPESCR
				1237.92	SIPINPLPACR
				1918.72	CDALSILVNGVITEDGSR
trypsin/amylase inhibitor pUP38	gi|225103	not mapped	12.4	1875.99	LLVAPGQCNLATIHNVR

10.2. Barley proteins identified after chymotryptic digestion

The summary of barley proteins identified after chymotryptic digestion in the entire doctoral thesis, including corresponding identified peptides and their sequences.

protein name	NCBInr entry	UniProtKB entry	mass [Da]	$[M + H]^+$ (observed)	peptide sequence
alpha-amylase inhibitor BMAI-1	gi|2506771	P16968	16.4	1506.82	DRAVASLPAVCNQY
alpha-amylase type B isozyme	gi|2851583	P04063	47.5	1003.51	RFDFAKGY
				1186.70	RVQKELVEW
				1461.80	GPRYDVGNLIPGGF
				1619.87	IDRSEPSFAVAEIW
alpha-amylase/trypsin inhibitor CMa	gi|585289	P28041	15.5	1569.84	KDLPGCPKEPQRDF
				1686.83	FIGRRSHPDWSVL
alpha-amylase/trypsin inhibitor CMb	gi|585290	P32936	16.5	1001.62	VRILVTPGF
				1388.78	VRILVTPGFCNL
				1458.71	ANIPQQCRCQAL
				1591.80	TATPITPLPSCRDY
alpha-amylase/trypsin inhibitor CMd	gi|585291	P11643	18.5	1812.91	VAPGQCNLATIHNVRY
				2164.93	CCQELAEIPQQCRCEAL
B1 Hordein	gi|82548225	Q2XQF0	34.5	1227.70	RHEAIRAIVY
				1372.65	YRILRGVGPSVGV
				1834.00	LQPHQIAQLEATTSIAL
B3 Hordein	gi|123459	P06471	30.2	920.55	RAIVYSIV
				1213.71	RHEAVRAIVY
				1487.62	RTLPTMCSVNVPL
				1501.79	RTLPTMCSVNVPL + Propionamide (C)
				1650.83	RTLPTMCSVNVPLY
				1666.83	RTLPTMCSVNVPLY + Oxidation (M)
				1834.00	LQPHQIAQLEATTSIAL
beta-amylase	gi|113786	P16098	59.6	1107.55	NDTPERTQF
				1254.63	NDTPERTQFF
				1420.78	RLSNQLVEGQNY
				1583.86	VDRMHANLPRDPY
				2183.27	SNNLIKHGDRILDEANKVF
				2257.22	KAAAAAVGHPEWEFPNDVGQY
beta-D-xylosidase	gi|18025342	Q8W011	84.4	1126.56	DGNPKYNRY
				1310.78	AKNNPKIGAIVW
				1518.87	RIGQVIGTEARGVY
				1618.93	YRIGQVIGTEARGVY
beta-glucosidase	gi|804656	Q40025	57.7	1021.51	GDRVKNWF
				1072.52	YRDYITEL
				1100.65	KIVGNRLPGF
				1188.62	NEPRVVAALGY
				1212.61	SRQGFPAGFVF
				1711.84	FLDPITNGRYPSSML
				1824.92	NRLIDYMLQQGITPY
				1906.06	IVTHNIILSHAAAVQRY
				2145.03	SRIFPDGTGKVNQEGVDYY
				2233.10	SADESRMVKGSIDYVGINQY
C Hordein	gi|167016	Q40037	5.9	1425.83	LPQKPFPVQQPF
				2131.09	RQQAELIIPQQPQQPFPL
				2156.13	IIPQQPQQPFPLQPHQPY
				2794.41	QPQQPFPQQPQQPLPRPQQPFPW
C Hordein	gi|442524	Q41210	36.5	1171.61	RQLNPSSQEL
				1425.78	LPQQPFPVQQPF
				1989.93	RQLNPSSQELQSPQQSY
				2103.76	RQLNPSSQELQSPQQSYL
				2156.13	IIPQQPQQPFPLQPHQPY
				2466.26	QPQQPFPQPQQPIAHQPQQPF
				2794.41	QPQQPFPQQPQQPLPRPQQPFPW
cold-regulated protein	gi|10799810	Q9FSI8	17.6	1725.92	ALVVQDIDADERVPW
D Hordein	gi|1167498	Q40054	75.1	879.51	SQVVRQY
				1172.61	LQPGQGQQGPY
				1398.78	RDVSPECRPVAL
				1729.92	TQQKPGQGYNPGGTSPL
				1816.94	DQQLVGQLPWSTGLQM + Oxidation (M)
				2054.15	EACRRVVDQQLVGQLPW
				2313.21	HQQGGGFGGGLTTEQPQGGKQPF
				2928.60	YPIATSPQQPGQGQQLGQGQQPGHGQQL
				4192.05	HQSVTSSQQPGQGQQGSYPGSTFPQQPGQGQQPG QRQPW

protein name	NCBInr entry	UniProtKB entry	mass [Da]	$[M + H]^+$ (observed)	peptide sequence
γ Hordein	gi|123464	P17990	34.7	1047.59	LQPHQIAQL
				1133.58	QQLNPCKVF
				1725.84	QPQQQPQFPQQKPF
				2687.88	QQPQHQFPQPTQQFPQRPLLPF
chain C, Amy2BASI PROTEIN-protein complex from barley seed	gi|4699833	P07596	22.5	872.41	RADANYY
				1049.55	RDLKGGAWF
				2127.07	VSQDPNGQHDGFPVRITPY
				2274.15	FVSQDPNGQHDGFPVRITPY
				2353.11	ADPPPVHDTDGHELRADANYY
predicted protein	gi|326523729	F2DD64	86.8	1310.78	AQNNPKIGAIVW
predicted protein	gi|326489434	F2E2X6	44.7	1294.65	DIYWPLRNAF
				1647.84	DAATSGIARADKVEPF
				2357.23	RAVGSCAPGELLESLPAGAAGVAGF
predicted protein	gi|326521432	F2DP98	48.3	1427.71	VQRSGALTHVVVY
				1468.78	HASPPEFRKRPF
				2095.18	SAAPSPALIPRDQPAVAAHIL
predicted protein	gi|326513840	F2CYL7	55.8	1017.55	VQVASRGGPF
				1095.59	RIPRYFPF
				1574.96	RVILGPELAAGLGVPL
				1639.75	NLFDHEPSFRNTY
				2308.12	SISVDKHDYEPLDRSDIGVY
predicted protein	gi|326498119	F2DIK1	33.8	935.56	VRDVATQF
				1404.83	DRTPEEILGIVY
				1782.14	VAGLLGVESAQDAVIRAL
				1839.10	SLAYDRTPEEILGIVY
predicted protein	gi|326529599	F2EBM4	76.9	2053.98	IQEGGSETSSLEVQRGDVY
protein z-type serpin	gi|1310677	P06293	43.3	1014.67	RALGLQLPF
				1518.88	VLANESSTGGPRIAF
				1614.96	ATDVRLSIAHQTRF
				1931.11	RLASAISSNPERAAGNVAF
				2115.26	ALRLASAISSNPERAAGNVAF
				2457.40	STEPEFIENHIPKQTVEVGRF
putative splicing factor 3b	gi|114318675	A7Y0E4	41.3	1215.58	SLDPDDCLQPL
serine carboxypeptidase I	gi|2815493	P07519	54.6	1634.91	AGYVTVDEGHGRNLF

10.3. Curriculum vitae

Personal information:

Name and surname:	**Dagmar Benkovská**
Date of birth:	8. 5. 1985
Nationality:	Czech
Maiden name:	Smětalová
E-mail:	benkovskad@gmail.com

Education:

2009 –	**PhD. Student – Doctoral studies in Food chemistry** Brno University of Technology, Faculty of Chemistry (FCH BUT), Institute of Food Chemistry and Biotechnologies, CR Aim of PhD. thesis: Barley proteomic studies related to beer production
2007 – 2009	**Master's degree study in Food chemistry and biotechnology, Ing.** FCH BUT, Institute of Food Chemistry and Biotechnologies, CR Diploma thesis: Determination of propionic acid in bakery products by liquid chromatography.
2004 – 2007	**Bachelor's degree study in Food chemistry, Bc.** FCH BUT, Institute of Food Chemistry and Biotechnologies, CR Bachelor's thesis: Additives in foods and their possible adverse effects.

Employment:

2009 –	Institute of Analytical Chemistry (IACH) of the Academy of Sciences of the Czech Republic, v.v.i. Department of Proteomics and Glycomics (since March 2013 Department of Bioanalytical Instrumentation); PhD. Student

Skills:

Analytical techniques:	Operation with 1D HPLC (Dionex, Agilent, Waters), MALDI-TOF/TOF MS (AB), TLC, SPE, 1D/2D SDS-PAGE
Languages:	English, German
Computer skills:	User knowledge of MS Office, MS Windows, basic knowledge of HTML
Driving licence:	Category B

Competition participations:

2010, IACH Brno	Competition of publications "Soutěž původních vědeckých prací a patentů 2010"
2011, IACH Brno	Competition of publications "Soutěž původních vědeckých prací a patentů 2010") – awarded with 5rd place
2012, FCH BUT	PhD. student competition within the student conference "Studentská odborná konference Chemie a společnost 2012" – awarded with 3rd place
2012, University of Pardubice	Jean-Marie Lehn chemistry award 2012

Pedagogical activities:

2009 – 2010, FCH BUT	Laboratory practice of instrumental and structural analysis (theme: HPLC analysis): MSc. students of 1st year Laboratory practice of food analysis: BSc. students of 3rd year

10.4. List of publications

LAŠTOVIČKOVÁ, M, MAZANEC, K., **BENKOVSKÁ, D.**, BOBÁĽOVÁ, J., Utilization of linear mode of MALDI-TOF mass spectrometry in the study of glycation during the malting process. *Journal of the Institute of Brewing* 2010, 116 (3), pp 245–250.

LAŠTOVIČKOVÁ, M, **SMĚTALOVÁ, D.**, BOBÁĽOVÁ, J., The Combination of Lectin Affinity Chromatography, Gel Electrophoresis and Mass Spectrometry in the Study of Plant Glycoproteome: Preliminary Insights. *Chromatographia* 2011, 73, Suppl. 1, pp 113–122.

BENKOVSKÁ, D., FLODROVÁ, D., PSOTA, V., BOBÁĽOVÁ, J., Vliv pivovarského procesu na profil proteinů ječmene/Influence of the brewing process on the barley protein profile. *Kvasný průmysl*. 2011, 57, 7-8, pp 260–265.

FLODROVÁ, D., ŠALPLACHTA, J., **BENKOVSKÁ, D.**, BOBÁLOVÁ, J., Application of proteomics to hordein screeening in the malting process. *European Journal of Mass Spectrometry*, 2012, 18, 3, pp 323–332.

ILLKOVÁ, K., ZEMKOVÁ, Z., FLODROVÁ, D., JÄGER, J., **BENKOVSKÁ, D.**, OMELKOVÁ, J., VADKERTIOVÁ, R., BOBÁLOVÁ, J., STRATILOVÁ, E., Production of Geotrichum candidum polygalacturonases via solid state fermentation on grape pomace. *Chemical Papers*, 2012, Roč. 66, č. 9, pp 852–860.

BENKOVSKA, D., FLODROVA, D., BOBALOVA, J., Application of monolithic affinity HPLC column for fast determination of malt glycoproteins. *Journal of Liquid Chromatography & Related Technologies* 2013, 36(5), pp. 561–572.

10.5. Conference participations

SMĚTALOVÁ, D., LAŠTOVIČKOVÁ, M, MAZANEC, K., BOBÁĽOVÁ, J., Proteomic approach for study of glycation during the malting process. *4th Central and Eastern European Proteomics Conference, Wien, Austria*. 2010

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