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BARLEY PROTEOMIC STUDIES RELATED TO BEER PRODUCTION

PROTEOMICKÉ STUDIE JEČMENE SOUVISEJÍCÍ S VÝROBOU PIVA

Syllabus of doctoral thesis

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SUMMARY

1 ABSTRACT .................................................................................................................. 5
2 INTRODUCTION ........................................................................................................ 6
3 THEORETICAL PART .................................................................................................. 7
  3.1 Proteomics ............................................................................................................. 7
    3.1.1 Protein post-translational modifications (PTMs) ............................................... 7
    3.1.2 Glycosylation of proteins ............................................................................... 7
  3.2 Beer production ..................................................................................................... 8
  3.3 Important barley proteins .................................................................................... 8
    3.3.1 Pathogenesis-related proteins (PRs) ................................................................. 8
    3.3.2 Hordeins ......................................................................................................... 9
4 EXPERIMENTAL ......................................................................................................... 10
  4.1 Samples and protein extraction .............................................................................. 10
  4.2 Proteins separation ................................................................................................ 10
    4.2.1 1D GE – SDS-PAGE ....................................................................................... 10
    4.2.2 Reversed phase C18 HPLC ............................................................................ 10
    4.2.3 HPLC affinity chromatography on ConA monolithic column ....................... 11
    4.2.4 ConA affinity chromatography ....................................................................... 11
  4.3 Protein enzymatic digestion .................................................................................. 11
    4.3.1 Digestion in-gel ............................................................................................... 11
    4.3.2 Digestion in-solution ...................................................................................... 12
  4.4 Isotopic labeling iTRAQ ....................................................................................... 12
  4.5 MALDI-TOF/TOF MS analysis ............................................................................. 12
5 AIMS OF THE THESIS ............................................................................................... 13
6 RESULTS AND DISCUSSION .................................................................................... 14
  6.1 Barley water-soluble proteins and their changes during individual stages of the malting and brewing process ........................................................................................................ 14
    6.1.1 Analysis of barley proteins by 1D gel electrophoresis .................................... 14
    6.1.2 Study of protein changes during the malting and brewing process by HPLC C18 separation ................................................................................................................................. 16
    6.1.3 Changes in low-molecular weight protein profile during malting and mashing ... 18
  6.2 Barley prolamins (hordeins) and their changes during malting ................................ 20
  6.3 Study of barley glycoproteins ............................................................................. 22
7 CONCLUSIONS .......................................................................................................... 26
8 ABBREVIATIONS ....................................................................................................... 27
9 REFERENCES ............................................................................................................. 28
10 CURRICULUM VITAE ................................................................................................ 30
11 LIST OF PUBLICATIONS ......................................................................................... 32
1 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.
2 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. 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.⁴,⁵ Malting is controlled germination of cereals evoking physical and biochemical changes within the grain, consequently stabilized by grain drying.⁵ 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.⁵,⁶ 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.⁶

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.⁷
3 THEORETICAL PART

3.1 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.\(^3,8\)

3.1.1 Protein post-translational modifications (PTMs)

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).\(^3\) PTMs play an important role in organism and represent 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.\(^3,9,10\)

3.1.2 Glycosylation of proteins

Glycosylation is the covalent linkage of an oligosaccharide side chain to a protein.\(^11\) 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.\(^12,10\)

A sugar moiety can be attached to a protein either during an enzymatic reaction, or a chemical reaction with no enzyme contribution.\(^12\)

The non-enzymatic glycosylation (glycation) of proteins take place in the process known as Maillard reaction. Glycation is a 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. This modification is involved in several age-related diseases in humans.\(^11,13,14,15\) The proteins in barley malt are known to be glycated by D-glucose, which is a product of starch degradation during malting.\(^14\)

Various forms of enzymatic glycosylation are known depending on the linkage between the protein backbone and the oligosaccharide moiety: \(N\)-glycosylation, \(O\)-glycosylation, \(C\)-mannosylation, and glycosylphosphatidylinositol anchor attachments.

In case of plants, \(N\)-glycosylation is the most studied protein modification.\(^16\) \(N\)-linked glycoproteins are expressed by all eukaryotic cells.\(^17\) Oligosaccharides are attached through \(\alpha\)-\(N\)-glycosidic bond to nitrogen of the amide group of asparagine
residues that are constituent of the potential $N$-glycosylation specific sequence $N$-X-S/T (serine or threonine, where $X$ is any amino acid except proline).\textsuperscript{10,18} $N$-glycans contain a common trimannosyl-chitobiose core $\text{Man}_3\text{GlcNAc}_2$ (mannose-$N$-acetyl-$d$-glucosamine) with one or more antennae attached to each of the two outer mannose residues.\textsuperscript{8}

### 3.2 BEER PRODUCTION

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. 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.\textsuperscript{5,19} Then, 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. Acquired wort is then cooled down and separated from sediment, thus prepared for the fermentation process. Wort fermentation is promoted by brewer’s yeast \textit{Saccharomyces cerevisiae} that cause the controlled conversion of saccharides into alcohol and CO$_2$ as well as production of required organoleptic properties of beer.\textsuperscript{19} Fermentation also tends to produce flavours that are considered undesirable in finish beer. For this reason, beer must undergo some form of maturation by the continuing action of the yeast, 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.\textsuperscript{20} In the end of the brewing process, beer is separated from yeast by sedimentation and filtered.\textsuperscript{19}

### 3.3 IMPORTANT BARLEY PROTEINS

Proteins and peptides contained in beer originate mainly from barley seeds.\textsuperscript{7} Proteins are essential for the quality of malt and beer. 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.\textsuperscript{5}

#### 3.3.1 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 and are classified into 17 families.\textsuperscript{4,7} 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.\textsuperscript{7}
**Protease/α-amylase inhibitors**

Protease inhibitors (PR-6) constitute the largest group of proteins identified in barley, malt and beer. These proteins are involved in plant defence by enzymes inhibition. Serine protease inhibitors (serpins) can be classified in the group of PR-6 proteins. Protein Z is the most abundant malt and beer protein and has been the first characterized protein in beer. Protein Z is glycated during the brewing process through Maillard reaction. 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. The proteolytic cleavage of protein Z leading to formation of the C-terminal 363–399 fragment is known, probably due to the interaction between protein Z and serine proteases.

**Non-specific lipid transfer proteins (ns-LTPs)**

Non-specific lipid transfer proteins (ns-LTPs; 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. They are subdivided into two families: ns-LTP1 and ns-LTP2. Non-specific LTP1 is an abundant protein of the aleurone layers from barley endosperm and has been studied more thoroughly than ns-LTP2. LTP1 is glycated during the malting process and 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. Although LTPs typically bind fatty acids in a non-covalent way, covalently modified forms of LTP1 were also identified. First known modified form, named LTP1b, was isolated from barley and beer extract and exhibit a molecular weight 294 Da higher than LTP1 (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.

Compared to ns-LTP1, ns-LTP2 has higher lipid transfer activity and is also more stable. It is glycated during the malting as well.

**3.3.2 Hordeins**

 Hordeins, alcohol-soluble prolamin fraction of barley proteins, are storage proteins of barley grain and the main protein fraction of barley endosperm. 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.
4 EXPERIMENTAL

4.1 SAMPLES AND PROTEIN EXTRACTION

Barley grain, grain from 1\textsuperscript{st} to 5\textsuperscript{th} day of malting, green malt and malt, as well as sweet wort, wort and green beer samples, were obtained from Czech Research Institute of Brewing and Malting, Brno. 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.

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% dithiothreitol (DTT). Extractions were carried out in the shaker for 30 min at the room temperature. The mixtures were centrifuged 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.

4.2 PROTEINS SEPARATION

4.2.1 1D GE – SDS-PAGE

Proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) 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). For gel electrophoresis, protein lyophilized extracts were dissolved in Laemmli sample buffer (mixed with β-mercaptoethanol in the ratio 15:1) and boiled for five minutes in a water bath. Separation was performed in running buffer at constant voltage. After gel electrophoresis, gels were fixed using 12\% trichloracetic acid, washed in water and the protein visualization was carried out overnight using Bio-Safe Coomassie stain.

4.2.2 Reversed phase C18 HPLC

All proteins separations by high performance liquid chromatography (HPLC) were performed on 1100 Series chromatograph equipped with diode array detector. The injection loop of 50 μL and sample concentration of 50 mg/mL was used. Proteins were detected using UV light at 214 nm.

Reverse phase C18 liquid chromatography separation was carried on C18 column Poroshell 300SB (2.1 x 7.5 mm, 5 μm; Agilent Technologies). 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.
4.2.3  HPLC affinity chromatography on ConA monolithic column

The enrichment of barley glycoproteins was performed on concanavalin A (ConA) ProSwift monolithic ConA-1S affinity column, 5 x 50 mm (Dionex). 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 dialysis cassettes with 3.5 kDa cut-off, concentrated in Speed-Vac and finally lyophilized.

4.2.4  ConA affinity chromatography

Affinity chromatography was performed on manually prepared columns packed with ConA bound to agarose. 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 MnCl₂ and 1 mmol/L CaCl₂. 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 was resuspended in 2 mL of loading buffer. First, 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, concentrated in SpeedVac and lyophilized.

4.3  PROTEIN ENZYMATIC DIGESTION

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

4.3.1  Digestion in-gel

Bands selected from polyacrylamide gel were excised and the in-gel enzymatic digestion was performed according to Jensen et al. protocol. Proteins were reduced by DTT and alkylated by iodoacetamide (IA). Proteins were digested with trypsin or chymotrypsin overnight at 37°C. Peptides were extracted from the gel and dried down in SpeedVac.
4.3.2 Digestion in-solution

Lyophilized proteins were re-dissolved in 50 mM ammonium bicarbonate. Proteins were reduced by DTT and alkylated by IA. 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% trifluoroacetic acid (TFA) and samples were dried down in SpeedVac.

4.4 ISOTOPIC LABELING ITRAQ

For the isobaric tags for relative and absolute quantification (iTRAQ), 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. Tris-(2-carboxyethyl) phosphine was used for protein reduction, and S-methyl methanethiosulfonate for protein alkylation.

Peptides were incubated with iTRAQ (m/z 114, 117) reagents at room temperature for 90 min. After labeling, the contents of both paired samples (samples that should to be compared) were mixed together 1:1 ratio and dried completely.

4.5 MALDI-TOF/TOF MS ANALYSIS

Prior to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS) analysis, the desalting, purification and concentration of peptides was performed using ZipTip C18 10 μL pipette tips. This purification consists of four main steps: hydration of the C18 resin, sample loading (dissolved in 0.1% TFA), washing of contaminants and sample elution (with 50% ACN in 0.1% TFA). 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.

Samples were mixed with matrix solution in the ratio 1:1 directly on the MALDI target. α-Cyano-4-hydroxycinnamic acid (CHCA) and 2,6-dihydroxyacetophenone (DHAP) were used as matrices for peptide and protein samples, respectively.

MALDI-TOF/TOF MS experiments were performed on Applied Biosystems 4700 Proteomics Analyzer, or on AB SCIEX TOF/TOF™ 5800 System (both from AB SCIEX). Linear positive mode (for proteins analysis only) or positive reflectron mode were utilized. Mass peak lists required for protein identification were created by Peaks to Mascot tool in 4000 (TOF/TOF) Series Explorer software. Acquired data were submitted to the Mascot database searching. NCBInr database was used for protein identification. Identified proteins were also searched in the UniProtKB database to obtain some additional information.
5 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 modifications of barley protein, 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.
6 RESULTS AND DISCUSSION

6.1 BARLEY WATER-SOLUBLE PROTEINS AND THEIR CHANGES DURING INDIVIDUAL STAGES OF THE MALTING AND BREWING PROCESS

Since investigated barley extracts represents a complex mixture of different water-soluble compounds as well as different kinds of proteins, it is necessary to separate this complex protein mixture before the protein MS identification. Various separation techniques were used. Moreover, the changes of low-molecular weight barley proteins were studied using the linear mode of MALDI-TOF MS.

6.1.1 Analysis of barley proteins by 1D gel electrophoresis

Water-extracted barley proteins 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 mass spectrometry. For the malting process investigation, samples of grain, 1st – 5th day of the malting process, green malt and malt were used. Furthermore, samples of sweet wort, wort and green beer were used for the investigation of brewing process. The Figure 1 shows SDS-PAGE separation of barley proteins and their changes during the malting and brewing. Proteins identified in marked bands are listed in the Table 1.

![Figure 1: SDS-PAGE separation of barley proteins from individual steps of the malting and brewing process. Protein bands above the marked lines were analyzed.](image)
Table 1: Summary of proteins identified in individual brewing stages. The presence of proteins in individual samples is highlighted by blue colour.\textsuperscript{31}

<table>
<thead>
<tr>
<th>spot No.</th>
<th>grain</th>
<th>malt</th>
<th>sweet wort</th>
<th>wort</th>
<th>green beer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td>predicted protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>beta-D-xylosidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td>beta-glucosidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td>beta-amylase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>chain A, Amy2BASI PROTEIN-Protein Complex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td>alpha-amylase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td>glyceraldehyde-3-phosphate dehydrogenase, cytosolic fructose-bisphosphate aldolase aldose reductase peroxidase BP 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td></td>
<td>glucose and ribitol dehydrogenase homolog</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td></td>
<td>26 kDa endochitinase 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td></td>
<td>26 kDa endochitinase 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td></td>
<td>triosephosphate isomerase, cytosolic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td></td>
<td>barperm1 thiaumatin-like protein TLP6; TLP7 basic pathogenesis-related protein PR5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td></td>
<td>thiaumatin-like protein TLP8 chitinase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td></td>
<td>pathogenesis-related proteins</td>
<td>alpha-amylase/trypsin inhibitor CMb barwin trypsin inhibitor CMe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td></td>
<td>alpha-amylase inhibitor BDAI-1 trypsin/amylase inhibitor pUP38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td></td>
<td>non-specific lipid-transfer protein 1 lipid transfer protein complexed with palmitate</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 indicate 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
barley grain. 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 is forming during the malting process
and was identified only in the malt sample. From the SDS-PAGE separation of
samples from individual stages of the malting process (figure not shown) it was
found that the content of β-amylase seemed to increase linearly during all days of
the malting process, and the first signs of α-amylase appeared on Coomassie stained
gel in the sample from the 2nd day of malting. The content of proteins in line No. 7
(protein Z, glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate
aldolase; see Figure 1 and Table 1) probably began to increase from the 4th day
of malting. The amount of protease/α-amylase inhibitors seemed to decrease during
malting.

At the beginning of the brewing process, malt is boiled in water leading to sweet
wort production (i.e. mashing process). The protein amount is significantly
decreasing during mashing 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; however, they were apparently
precipitated during brewing. In contrast to α-amylase, more temperature-sensitive
β-amylase was denatured during mashing.

In sweet wort, wort and green beer samples, two general protein areas are evident,
namely area about 40 kDa and area about 20 to 6 kDa. In these areas, protein Z and
several low-molecular weight proteins (protease/α-amylase inhibitors, ns-LTP) were
identified. While protein Z content seems to be stable during the whole brewing
process, the content of low-molecular weight proteins is decreasing. Obtained results
confirmed that identified barley proteins (belonging to the group of PRs) 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.

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).

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

The changes in protein profile during the malting and brewing process are shown
in chromatograms obtained after reversed phase C18 HPLC separation of barley
grain, malt, sweet wort, wort and green beer samples (Figure 2). 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 2. In the first and second fraction, ns-LTP2 and ns-LTP1b were detected using the linear mode of MALDI-TOF MS, respectively.

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 1). 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 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.

![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.](image)

Figure 2: 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.
Table 2: Proteins identified in barley grain and malt after reverse phase C18 HPLC separation, tryptic in-solution digestion and MALDI-TOF/TOF MS analysis.

<table>
<thead>
<tr>
<th>peak No.</th>
<th>grain</th>
<th>malt</th>
<th>NCBInr entry</th>
<th>UniProtKB entry</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>alpha-amylase/trypsin inhibitor CMb</td>
<td>gi</td>
<td>585290</td>
<td>P32936</td>
</tr>
<tr>
<td></td>
<td>trypsin/amyase inhibitor pUP38</td>
<td>gi</td>
<td>225103</td>
<td>not mapped</td>
</tr>
<tr>
<td></td>
<td>alpha-amylase/trypsin inhibitor CMd</td>
<td>gi</td>
<td>585291</td>
<td>P11643</td>
</tr>
<tr>
<td></td>
<td>CMd3 protein</td>
<td>gi</td>
<td>2264392</td>
<td>O24000</td>
</tr>
<tr>
<td></td>
<td>alpha-amylase inhibitor BMAI-1</td>
<td>gi</td>
<td>2506771</td>
<td>P16968</td>
</tr>
<tr>
<td>4</td>
<td>bifunctional alpha-amylase/subtilisin inhibitor chain C, Amy2BASI PROTEIN-protein complex from barley seed</td>
<td>gi</td>
<td>18916</td>
<td>F2E8J4</td>
</tr>
<tr>
<td>5</td>
<td>putative avenin-like a precursor</td>
<td>gi</td>
<td>326501830</td>
<td>F2EGD5</td>
</tr>
<tr>
<td>6</td>
<td>subtilisin-chymotrypsin inhibitor-2A</td>
<td>gi</td>
<td>124122</td>
<td>P01053</td>
</tr>
<tr>
<td></td>
<td>chymotrypsin inhibitor-2</td>
<td>gi</td>
<td>158530106</td>
<td>A8V4D2</td>
</tr>
<tr>
<td>7</td>
<td>26 kDa endochitnine 2 chain A, the refined crystal structure of an endochitnine</td>
<td>gi</td>
<td>116316</td>
<td>P23951</td>
</tr>
<tr>
<td>8</td>
<td>aldose reductase chain A, crystal structure of barley grain peroxidase 1</td>
<td>gi</td>
<td>1157834680</td>
<td>P23951</td>
</tr>
<tr>
<td>9</td>
<td>fructose-bisphosphate aldolase</td>
<td>gi</td>
<td>226316443</td>
<td>C1J960</td>
</tr>
<tr>
<td>10</td>
<td>alpha-amylase type B isozyme</td>
<td>gi</td>
<td>2851583</td>
<td>P04063</td>
</tr>
<tr>
<td>11</td>
<td>beta-amylase</td>
<td>gi</td>
<td>10953877</td>
<td>Q9FUK6</td>
</tr>
<tr>
<td>12</td>
<td>beta-glucosidase</td>
<td>gi</td>
<td>804656</td>
<td>Q40025</td>
</tr>
<tr>
<td></td>
<td>protein z-type serpin</td>
<td>gi</td>
<td>1310677</td>
<td>P06293</td>
</tr>
</tbody>
</table>

In the HPLC C18 chromatogram of sweet wort sample, disappearance of some peaks was obvious because of protein denaturation during mashing. 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 the identification after SDS-PAGE, both of these samples contain ns-LTPs, protein Z and other protease/α-amylase inhibitors.

The reversed phase C18 HPLC separation of barley proteins represents a suitable method for rapid barley protein separation. 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.

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

The changes of low-molecular weight barley proteins during malting and mashing were studied by MALDI-TOF mass spectrometer in the linear mode. Acquired MALDI-TOF spectra of barley grain, malt and sweet wort labelled with names of corresponding proteins are shown in Figure 3.

Following proteins belong among major proteins in the low-mass area: protein Z fragment (C-terminal 363 – 399 fragment; 4.03 kDa), LTP1 (9.69 kDa), LTP2 (7.10 kDa) and LTP1b (LTP1 with bounded 294 Da lipid-like molecule 9-hydroxy-10-oxo-12(Z)-octadecenoic acid; 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. From the analysis of individual steps of the malting process it was found that fragment of protein Z was detected from the 3rd day of the malting process (Figure 4). 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.

![Graph](image.png)

Figure 3: Linear mode MALDI-TOF 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 (●).

All discussed proteins are non-enzymatically glycated during the malting process (Figure 4). A hexose unit (very likely glucose) is bound to the protein, which leads to the increase of the molecular mass about 162 Da. 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. Protein Z fragment was glycated with one hexose unit and this modification was slightly detected from the 4th day of the malting process.
Low-molecular weight barley proteins and the modifications they have undergone during the malting and mashing can be successfully monitored using the MALDI MS in the TOF linear mode.

6.2 BARLEY PROLAMINS (HORDEINS) AND THEIR CHANGES DURING MALTING

Barley prolamins (hordeins) are alcohol-soluble storage proteins. They were separated by SDS-PAGE on 12% polyacrylamide gel (Figure 5), in-gel digested with chymotrypsin and analyzed by MALDI-TOF/TOF MS. Identified proteins are shown in Table 3.
In contrast to various proteins found in the albumin fraction, proteins in alcohol-soluble prolamin fraction can be divided in only four general groups: D hordeins, C hordeins, B hordeins and γ hordeins. According to the spot intensities on the gel, B and C hordeins are the major proteins. 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 No. 1 corresponding to D hordein.

Table 3: Identified proteins from barley prolamin fraction

<table>
<thead>
<tr>
<th>spot No.</th>
<th>protein</th>
<th>NCBI entry</th>
<th>UniProtKB entry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>D hordein</td>
<td>gi</td>
<td>1167498</td>
</tr>
<tr>
<td>2.</td>
<td>C hordein</td>
<td>gi</td>
<td>167016</td>
</tr>
<tr>
<td>3.</td>
<td>C hordein</td>
<td>gi</td>
<td>442524</td>
</tr>
<tr>
<td>4.</td>
<td>C hordein</td>
<td>gi</td>
<td>442524</td>
</tr>
<tr>
<td>5.</td>
<td>B1 hordein</td>
<td>gi</td>
<td>82548225</td>
</tr>
<tr>
<td>6.</td>
<td>B3 hordein</td>
<td>gi</td>
<td>123459</td>
</tr>
<tr>
<td>7.</td>
<td>γ hordein</td>
<td>gi</td>
<td>123464</td>
</tr>
</tbody>
</table>

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. Obtained data showed that the amount of C hordein is significantly decreasing during the malting process. The amount of C hordein in malt represented 35 % of the initial amount in barley grain (Figure 6).
Figure 6: Relative representation of C hordein during individual stages of the malting process. The amount of C hordein is decreasing during malting up to 35% of the initial quantity.32

6.3 STUDY OF BARLEY GLYCOPROTEINS

The enrichment of glycoproteins from a complex mixture is required for their analysis. For this purpose, the affinity chromatography with ConA lectin was used.

Figure 7: SDS-PAGE separation of the ConA AC unbound and bound fractions of barley grain and malt. Protein bands from the bound fractions above the marked lines were analyzed.

First, the barley grain and malt proteins were separated on affinity column manually filled with ConA bound to agarose. The obtained bound and unbound fractions were dialyzed and separated by SDS-PAGE (Figure 7). From gels of both barley grain and malt it is evident, that the majority of proteins occurred in the unbound fraction and minor glycosylated proteins were found in the bound fraction.
Table 4: Proteins identified in barley grain and malt after ConA affinity chromatography and SDS-PAGE separation. The amino acids (AA) triplet of possible N-glycosylation sites (N-X-S/T; X ≠ P) is listed in the table as well.

<table>
<thead>
<tr>
<th>spot No.</th>
<th>grain</th>
<th>malt</th>
<th>UniProtKB entry</th>
<th>possible N-glycosylation sites</th>
<th>quantity</th>
<th>position (AA triplet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td></td>
<td>predicted protein</td>
<td>F2DV95</td>
<td>8x</td>
<td>92 (NDT); 157 (NAT); 217 (NVT); 360 (NLT); 366 (NET); 502 (NGT); 596 (NYS); 720 (NTS)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>beta-D-xylosidase</td>
<td>Q8W011</td>
<td>4x</td>
<td>203 (NSS); 432 (NAS); 473 (NVS); 710 (NAT)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>purple acid phosphatase isoform a</td>
<td>C4PKL2</td>
<td>8x</td>
<td>140 (NYT); 205 (NTT); 236 (NGT); 292 (NKT); 414 (NYT); 465 (NFT); 500 (NET); 536 (NNT)</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td></td>
<td>predicted protein</td>
<td>F2DJN8</td>
<td>7x</td>
<td>30 (NTS); 58 (NNS); 273 (NVT); 465 (NIT); 475 (NFS); 523 (NAS); 727 (NKT)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>predicted protein</td>
<td>F2CYL7</td>
<td>3x</td>
<td>228 (NTT); 371 (NLT); 405 (NGS)</td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td></td>
<td>beta-amylase</td>
<td>Q9FUK6</td>
<td>4x</td>
<td>237 (NDT); 249 (NGT); 338 (NFT); 402 (NQS)</td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td></td>
<td>serine carboxypeptidase II</td>
<td>P08818</td>
<td>7x</td>
<td>148 (NTS); 159 (NRT); 291 (NIS); 341 (NVT); 347 (NYT); 352 (NCS); 472 (NVT)</td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td></td>
<td>concanavalin A</td>
<td>P02866</td>
<td>6x</td>
<td>79 (NVT)</td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td></td>
<td>germin B</td>
<td>Q9FYY4</td>
<td>1x</td>
<td>79 (NVT)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>germin F</td>
<td>Q9FYY3</td>
<td>1x</td>
<td>79 (NVT)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>germin D</td>
<td>Q9FYY2</td>
<td>1x</td>
<td>78 (NVT)</td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td></td>
<td>alpha-amylase inhibitor BMAI-1</td>
<td>P16968</td>
<td>1x</td>
<td>125 (NGT)</td>
</tr>
</tbody>
</table>

Proteins identified in the grain and malt bound fractions after digestion and MALDI-TOF/TOF MS analysis are summarized in the Table 4. 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 asparagine residue. Nevertheless, the real possibility of N-glycosylation site occupancy is difficult to find out without knowing the crystallographic structure of the protein or without direct study of glycopeptides or glycans after deglycosylation. Some of the barley grain and malt proteins identified in the bound fraction are even proven glycoproteins according to database^30^ (serine carboxypeptidase II and α-amylase inhibitor BMAI-1).

Despite the fact that many barley malt and grain glycoproteins were identified after this separation, 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.
To avoid these problems, glycoprotein enrichment using ProSwift ConA-1S monolithic HPLC column was performed. This column did not evince problem with ConA bleeding, moreover, separation showed also several additional improvements: for example a very good reproducibility, separation was faster, some operations were automated, and separated proteins were directly detected.

![Figure 8: 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.](image)

Table 5: Proteins identified in barley grain after HPLC ConA and SDS-PAGE separation. The amino acids triplet of possible N-glycosylation sites (N-X-S/T; X ≠ P) is listed in the table as well.

<table>
<thead>
<tr>
<th>spot No.</th>
<th>name of identified protein</th>
<th>UniProtKB entry</th>
<th>quantity</th>
<th>possible N-glycosylation sites position (AA triplet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>beta-glucosidase</td>
<td>Q40025</td>
<td>3x</td>
<td>86 (NGT); 356 (NQT); 423 (NVS)</td>
</tr>
<tr>
<td>2.</td>
<td>predicted protein</td>
<td>F2E2X6</td>
<td>4x</td>
<td>247 (NIT); 245 (NWT); 359 (NQT); 422 (NFT)</td>
</tr>
<tr>
<td></td>
<td>predicted protein</td>
<td>F2DP98</td>
<td>3x</td>
<td>45 (NSS); 187 (NPT); 267 (NVT)</td>
</tr>
<tr>
<td>3.</td>
<td>predicted protein</td>
<td>F2DIK1</td>
<td>2x</td>
<td>85 (NLT); 151 (NTT)</td>
</tr>
<tr>
<td>4.</td>
<td>predicted protein</td>
<td>F2CYL7</td>
<td>3x</td>
<td>228 (NTT); 371 (NLT); 405 (NGS)</td>
</tr>
<tr>
<td>5.</td>
<td>alpha-amylase inhibitor BMAI-1</td>
<td>P16968</td>
<td>1x</td>
<td>125 (NGT)</td>
</tr>
<tr>
<td>6.</td>
<td>alpha-amylase/trypsin inhibitor CMb</td>
<td>P32936</td>
<td>1x</td>
<td>124 (NLT)</td>
</tr>
</tbody>
</table>
SDS-PAGE separations of barley grain and malt bound and unbound fractions are shown in Figure 8. Proteins identified in barley grain and in barley malt are summarized in the Table 5 and Table 6, respectively.

Table 6: Proteins identified in barley malt after HPLC ConA and SDS-PAGE separation. The amino acids triplet of possible N-glycosylation sites (N-X-S/T; \(X \neq P\)) is listed in the table as well.\(^{33}\)

<table>
<thead>
<tr>
<th>spot No.</th>
<th>name of identified protein</th>
<th>UniProtKB entry</th>
<th>quantity</th>
<th>possible N-glycosylation sites position (AA triplet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>beta-D-xylosidase</td>
<td>Q8W011</td>
<td>4x</td>
<td>203 (NSS); 432 (NAS); 473 (NVS); 710 (NAT)</td>
</tr>
<tr>
<td></td>
<td>predicted protein</td>
<td>F2DD64</td>
<td>6x</td>
<td>41 (NYT); 146 (NET); 462 (NAT); 521 (NMS); 539 (NQT); 660 (NFS)</td>
</tr>
<tr>
<td>2.</td>
<td>beta-amylase</td>
<td>P16098</td>
<td>4x</td>
<td>237 (NDT); 249 (NGT); 338 (NFT); 402 (NQS)</td>
</tr>
<tr>
<td></td>
<td>beta-glucosidase</td>
<td>Q40025</td>
<td>3x</td>
<td>86 (NGT); 356 (NQT); 423 (NVS)</td>
</tr>
<tr>
<td>3.</td>
<td>alpha-amylase type B isozyme</td>
<td>P04063</td>
<td>1x</td>
<td>372 (NES)</td>
</tr>
<tr>
<td>4.</td>
<td>protein z-type serpin</td>
<td>P06293</td>
<td>2x</td>
<td>93 (NES); 170 (NTT)</td>
</tr>
<tr>
<td>5.</td>
<td>predicted protein</td>
<td>F2DIK1</td>
<td>2x</td>
<td>85 (NLT); 151 (NTT)</td>
</tr>
<tr>
<td>6.</td>
<td>serine carboxypeptidase I</td>
<td>P07519</td>
<td>3x</td>
<td>148 (NVS); 262 (NAT); 407 (NLT)</td>
</tr>
<tr>
<td>7.</td>
<td>predicted protein</td>
<td>F2EBM4</td>
<td>5x</td>
<td>140 (NAT); 222 (NWT); 300 (NLT); 426 (NGS); 469 (NTT)</td>
</tr>
<tr>
<td>8.</td>
<td>alpha-amylase/subtilisin inhibitor precursor (BASI)</td>
<td>P07596</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td>cold-regulated protein</td>
<td>Q9FSI8</td>
<td>1x</td>
<td>73 (NIS)</td>
</tr>
<tr>
<td>8.</td>
<td>alpha-amylase/trypsin inhibitor CMd</td>
<td>P11643</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>9.</td>
<td>putative splicing factor 3b</td>
<td>A7Y0E4</td>
<td>1x</td>
<td>235 (NET)</td>
</tr>
<tr>
<td>9.</td>
<td>alpha-amylase inhibitor BMAI-1</td>
<td>P16968</td>
<td>1x</td>
<td>125 (NGT)</td>
</tr>
<tr>
<td>9.</td>
<td>alpha-amylase/trypsin inhibitor CMb</td>
<td>P32936</td>
<td>1x</td>
<td>124 (NLT)</td>
</tr>
<tr>
<td>9.</td>
<td>alpha-amylase/trypsin inhibitor CMa</td>
<td>P28041</td>
<td>N/A</td>
<td>-</td>
</tr>
</tbody>
</table>

In barley grain, all identified proteins have at least one potential N-glycosylation site in their sequence. Nevertheless, not all proteins identified in the ConA retained fraction are possible N-glycoproteins. As a possible explanation, also non-enzymatically glycated proteins, 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.\(^{34}\)

Some of the identified barley grain and malt proteins are proven glycoproteins according to database\(^{30}\) (\(\alpha\)-amylose inhibitor BMAI-1, \(\alpha\)-amylose/trypsin inhibitor CMb, \(\alpha\)-amylose/trypsin inhibitor CMa, serine carboxypeptidase I). 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.
7 CONCLUSIONS

The barley grain proteins are significantly changed during the malting and brewing process. The aim of this dissertation 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 enzymatically forming N-glycosylations and non-enzymatically forming glycations 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. Conversely, during mashing, the protein amount is significantly decreasing. 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 performed using several separation techniques. 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 efficient identification of barley proteins as well as for rapid monitoring of changes in the protein profile during individual steps of the malting and brewing process. 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.

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 and the possibility of estimating the level of glycations of barley low-molecular weight proteins within the malting process.

In addition to water-soluble proteins, barley prolamins (hordeins) were studied as well. SDS-PAGE separation of alcohol-extracted proteins 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 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. First, a manually filled column with ConA-agarose was used. Nevertheless, ConA was bleeding from this column, which made the analysis more difficult. Therefore, it was replaced by monolithic HPLC column with firmly bound stationary phase in the next studies. After ConA enrichment, several potential glycoproteins and also some proven glycoproteins (according to the database) were identified in grain and malt samples.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>α-MMP</td>
<td>methyl-α-D-mannopyranoside</td>
</tr>
<tr>
<td>CHCA</td>
<td>α-Cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>ConA</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DHAP</td>
<td>2,6-dihydroxyacetophenone</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IA</td>
<td>iodoacetamide</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>isobaric tags for relative and absolute quantification</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>Man&lt;sub&gt;3&lt;/sub&gt;GlcNAc&lt;sub&gt;2&lt;/sub&gt;</td>
<td>mannose-N-acetyl-d-glucosamine</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>N</td>
<td>asparagine</td>
</tr>
<tr>
<td>ns-LTPs</td>
<td>non-specific lipid transfer proteins</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>PRs</td>
<td>pathogenesis related proteins</td>
</tr>
<tr>
<td>S</td>
<td>serine</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>T</td>
<td>threonine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight</td>
</tr>
</tbody>
</table>
9 REFERENCES

5. GUPTA, M., ABU-GHANNAM, N., GALLAGHAR E., Barley for Brewing: Characteristic Changes during Malting , Brewing and Applications of its By-Products. *Comprehensive Reviews In Food Science And Food Safety* 2010, 9, pp 318–328.
10 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:

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<th>Year</th>
<th>Organization</th>
<th>Description</th>
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<tr>
<td>2010</td>
<td>IACH Brno</td>
<td>Competition of publications “Soutěž původních vědeckých prací a patentů 2010”</td>
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<td>2011</td>
<td>IACH Brno</td>
<td>Competition of publications “Soutěž původních vědeckých prací a patentů 2010”) – awarded with 5&lt;sup&gt;th&lt;/sup&gt; place</td>
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<td>2012</td>
<td>FCH BUT</td>
<td>PhD. student competition within the student conference “Studentská odborná konference Chemie a společnost 2012” – awarded with 3&lt;sup&gt;rd&lt;/sup&gt; place</td>
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<td>2012</td>
<td>University of Pardubice</td>
<td>Jean-Marie Lehn chemistry award 2012</td>
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### Pedagogical activities:

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<td>2009 – 2010</td>
<td>FCH BUT</td>
<td>Laboratory practice of instrumental and structural analysis (theme: HPLC analysis): MSc. students of 1&lt;sup&gt;st&lt;/sup&gt; year</td>
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<td>Laboratory practice of food analysis: BSc. students of 3&lt;sup&gt;rd&lt;/sup&gt; year</td>
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11 LIST OF PUBLICATIONS


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

FLODROVÁ, D., SMĚTALOVÁ, D., ŠALPLAČKA, J., BOBÁĽOVÁ, J., Influence of various stationary phases on HPLC separation of glyced intact barley proteins. 28th International Symposium on Chromatography, Valencia, Spain. 2010

LAŠTOVIČKOVÁ, M., SMĚTALOVÁ, D., BOBÁĽOVÁ, J., Glycoproteome study of barley grain and malt: Preliminary insights. 16th International Symposium on Separation Science, Rome, Italy. 2010


BENKOVSKÁ, D., FLODROVÁ, D., BOBÁĽOVÁ, J., Concanavalin A lectin-based affinity chromatography for enrichment and determination of glycoproteins in barley malt. Chemistry and life, Brno. 2011. Published in: Chemické listy 105 (S)


BENKOVSKÁ, D., FLODROVÁ, D., BOBÁĽOVÁ, J., Changes of Protein Profile during the Brewing Process. Studentská odborná konference Chemie a společnost, Brno. 2012

FLODROVÁ, D., BENKOVSKÁ, D., BOBÁĽOVÁ, J., Sledování kvalitativních a kvantitativních změn proteomu ječmene na úrovni odrůdových rozdílů a změn v důsledku sladování. XLII. Symposia o nových směrech výroby a hodnocení potravin, Skalský Dvůr u Bystřice nad Pernštejnem. 2012.


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