JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

# Exploration of Beer Proteome Using OFFGEL Prefractionation in Combination with Two-Dimensional Gel Electrophoresis with Narrow pH Range Gradients

Hana Konečná,[†] Lukáš Müller,[§] Hana Dosoudilová,[†,§] David Potěšil,[†] Jana Buršíková,[†] Ondrej Šedo,[†] Ivana Márová,[§,⊗] and Zbyněk Zdráhal*[,†]

[†]Core Facility - Proteomics, Central European Institute of Technology, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic

[§]Department of Food Chemistry and Biotechnology and [⊗]Centres for Materials Research, Faculty of Chemistry, Brno University of Technology, Purkynova 118, 612 00 Brno, Czech Republic

Supporting Information

**ABSTRACT:** Two-dimensional gel electrophoresis in combination with mass spectrometry has already been applied successfully to study beer proteome. Due to the abundance of protein Z in beer samples, prefractionation techniques might help to improve beer proteome coverage. Proteins from four lager beers of different origins were separated by two-dimensional electrophoresis (2-DE) followed by tandem mass spectrometric analysis. Initially 52 proteins mostly from *Hordeum vulgare* (22 proteins) and *Saccharomyces* species (25 proteins) were identified. Preparative isoelectric focusing by OFFGEL Fractionator was applied prior to 2-DE to improve its resolution power. As a result of this combined approach, a total of 70 beer proteins from *Hordeum vulgare* (30 proteins), from *Saccharomyces* species (31 proteins), and from other sources (9 proteins) were identified. Of these, 37 proteins have not been previously reported in beer samples.

**KEYWORDS:** *beer proteome, two-dimensional gel electrophoresis, OFFGEL prefractionation, mass spectrometry, Hordeum vulgare, Saccharomyces species*

## INTRODUCTION

Beer is one of the most widespread beverages all around the world. The healthful and nutritive properties of beer have been recognized for thousands of years. Detailed beer characterization by current analytical techniques may significantly contribute to assessment of beer quality parameters and applied brewing technologies, as well as health aspects. Especially, with increased interest in health hazards, the demand for characterization of all beer components becomes a considerable issue. Czech beer is considered to be unique because of specific brewing technologies, so the European Union (EU) approved beer originating from the Czech and Moravian region to bear the "Czech Beer" trademark in October 2008. Therefore, modern instrumental methods enabling comprehensive characterization of beer composition may play an important role in trademark protection as well.

Proteins as an important class of beer components contribute significantly to final beer taste, foam stability, and haze formation, but their comprehensive characterization is not widely accomplished and generally still not satisfactory. Conclusions of previous studies concerning impacts of particular proteins are summarized in the literature,[1−7] and several inconsistent results were stated, for example, concerning the occurrence of barley hordeins in beer, which can potentially trigger the allergic response known as celiac disease.[8] Incomplete knowledge on the spectrum of beer proteins results in unsatisfactory understanding of protein influence on beer quality because beer quality features are orchestrated by concurrence of several proteins and various factors connected with beer technology process.[1]

Beer proteome analysis by two-dimensional gel electrophoresis followed by mass spectrometry has been successfully performed[1−6,8] and might significantly help to identify novel beer proteins, which could serve as beer quality markers.

Even though proteins are mostly removed or degraded to amino acids and small peptides during the malting and brewing processes,[1] proteins in the final beer originate predominantly from barley and from damaged yeast cells. All proteins detected in beer may be grouped into six main categories: protein Z isoforms, lipid transfer proteins (LTPs), trypsin/amylase inhibitors, hordeins, proteins from *Saccharomyces*, and other proteins.[1]

Previous studies dealing with beer proteome analysis indicate that barley cultivars and the malting process influence the protein profiles.[9−12] In a recent paper,[1] Japanese beer samples from five different barley cultivars and protein isolates obtained from three barley cultivars processed under different malting conditions were analyzed by two-dimensional electrophoresis (2-DE) for their proteomes. The aim of this study was to assess the relationships between particular barley cultivars and malting conditions, protein composition, and resulting beer quality. The list of identified spots in the beer proteome map involved 29 items. Of these a total of 12 proteins (9 of them from *Hordeum vulgare*) were identified by mass spectrometry. Barley proteins

Received: June 14, 2011
Revised: February 20, 2012
Accepted: February 21, 2012
Published: February 21, 2012





dx.doi.org/10.1021/jf204475e | J. Agric. Food Chem. 2012, 60, 2418−2426

included Z proteins, trypsin/amylase inhibitors, BDAI-1, pUP13, CMb, subtilisin−chymotrypsin inhibitor, and the lipid transfer protein LTP1. Yeast proteins detected were thioredoxin, enolase, triosephosphate isomerase, and phosphorelay protein. The gels were silver stained, enabling only a rough estimate of quantitative differences between individual beer samples. Several proteins on the 2-DE gel were often found in multiple spots. It is assumed that beer contains many isoforms or modified forms of proteins (particularly glycosylated forms, proteolytic cleavage products, and disulfide bond reduction products). This fact may complicate potential utilization of proteome analysis for beer quality and authenticity assessment. Thus, more detailed characterization of beer proteins is necessary, which requires further development of separation procedures and application of sophisticated detection methods.

As another crucial feature, the abundance of Z proteins may limit the detection of low-abundance proteins in a corresponding region of the 2-DE gel. In the case of low sample loads minor protein components are lost due to their amounts being under the detection limits of gel staining. On the other hand, higher loads of sample will result in a completely overloaded zone around protein Z, disabling detection of low-abundance proteins in this zone. Narrow p*I* range IPG (immobilized pH gradient) strip technology increases protein resolution by extending the narrow gradient size. However, protein loading limits on strips may complicate the detection of many low-abundance proteins in complex samples. Prefractionation of proteins according to their p*I* prior to 2-DE makes the application of preparative loads of protein to the narrow-range strips possible, thus improving separation and detection of both abundant and minor proteins.[13,14] Improved separation is also necessary for quantitative evaluation as one protein per spot is required.

A different approach for solving the problem with the abundant proteins was preferred by Righetti's group.[15] They incorporated the ProteoMiner-like prefractionation step with combinatorial peptide ligand libraries (CPLL) tailored to beer proteins prior to gel electrophoretic separation into the protocol. The aim of the process was to achieve a reduced dynamic range of protein concentrations while maintaining representatives of all proteins within the original sample. After saturation of their ligands, the excess of high-abundance proteins was washed away unbound. In contrast, the medium- and low-abundance proteins were concentrated completely. Eluted proteins were separated by SDS-PAGE and identified subsequently by mass spectrometry. Using this approach, a total of 20 different barley proteins, 40 proteins from *Saccharomyces cerevisiae*, 2 proteins from other *Saccharomyces* species, and 2 proteins from maize were identified.

In this study isoelectric focusing of beer proteins in solution followed by two-dimensional gel electrophoresis with utilization of narrow IPG strips combined with tandem mass spectrometry was applied for the characterization of beer proteins. Proteome maps of four lagers of different origins are presented.

## MATERIALS AND METHODS

**Beer Sample Preparation.** Four lager beers were provided by a local supplier: sample A, Pilsner Urquell (Czech Republic); sample B, Stella Artois (Belgium); sample C, Budweiser Budvar (Czech Republic); and sample D, Heineken (The Netherlands). Several methods of protein isolation from beer samples were compared, including three protein precipitation procedures and two variants of low molecular weight component removal by gel filtration on Sephadex G-25. Degassed raw beer samples were used as the starting material for all of the precipitation and desalting procedures. Precipitation protocols included acetone precipitation (20% TCA with 0.2% DTT in cold acetone at −20 °C, overnight), ethanol precipitation (cold ethanol at −20 °C, overnight), and the ProteoExtract Protein Precipitation Kit (Calbiochem). Sephadex G-25 disposable gravity-flow columns NAP-25 (GE Healthcare) and liquid chromatographic fractionation on the HiTrap Desalting column 1.6 × 2.5 cm (GE Healthcare) with the same sorbent utilizing water as eluting solvent were used. Preparative gel chromatography ran on the Perfusion Chromatograph BioCAD 700E Workstation (Applied Biosystems). Sample elution by water (flow rate = 1 mL/min) was monitored by UV absorption at 280 nm; only fractions with protein content were collected. Conductivity was monitored as well to prove that ions were really separated from protein fraction. Protein concentration in the degassed raw and desalted beer samples was quantified according to the Bradford protein assay (Bio-Rad) with BSA as a standard. Finally, all samples were vacuum-dried using the Savant SPD111 V vacuum concentrator (Thermo Scientific).

The application of the commercially available ProteoMiner Protein Enrichment Kit (Bio-Rad) for reduction of protein Z abundance and enrichment of the low-abundance proteins was evaluated for both raw beer and precipitated beer proteins. The kit was used according to the manufacturer's instructions. Proteominer-prefractionated beer samples were further processed by 2-DE.

**OFFGEL Prefractionation.** The OFFGEL High Resolution kit pH 3−10 (Agilent Technologies) was used for p*I*-based protein preparative isoelectric focusing (IEF) in solution. Protein samples (5 mg of whole protein, representing about 22 mL of raw beer) were (after degassing, desalting, and complete evaporation of water) solubilized in a Protein OFFGEL fractionation buffer supplied by the manufacturer (containing urea, thiourea, DTT, glycerol, and buffer with ampholytes), and aliquots were evenly distributed in a 24-well 3100 OFFGEL Fractionator (Agilent Technologies) tray according to supplier instructions: preset program OG24PR00 (separation limits: 8000 V, 200 mW, and 50 μA; starting voltage, 200−350 V; ending voltage, 2000−4200 V; after the application of 64 kVh, the protein separation zones were maintained at constant voltage). The liquid fractions were recovered, and each three sequential wells were pooled (i.e., fractions 1−3, 4−6, 7−9, 10−12, 13−15, 16−18, 19−21, and 22−24 were combined into eight pooled fractions marked I−VIII) and applied on the HiTrap desalting column to remove OFFGEL buffer components prior to 2-DE. Vacuum-dried pooled fractions (eight in total) were dissolved in the isoelectric focusing buffer (IPG buffer) and analyzed as described below.

**Isoelectric Focusing (First Dimension of 2-DE).** Vacuum-dried desalted protein samples were solubilized in the IPG buffer containing 7 M urea, 2 M thiourea, 2% w/v 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 60 mM dithiothreitol (DTT), 0.8% Biolyte 3/10 Ampholyte (Bio-Rad), and 0.003% bromophenol blue. Solubilized protein samples were centrifuged at 20000 rcf for 60 min at 10 °C before application to 18 cm ReadyStrip IPG strips pH 3−10NL (Bio-Rad) by passive rehydration (600 μg of whole beer protein in 360 μL of IPG buffer per IPG strip). IEF was performed in triplicate for each beer brand in the Protean IEF Cell (Bio-Rad) for 80000 Vh. Prior to the second dimension, the IPG strips were equilibrated for 10 min in the equilibration buffer (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2% DTT), followed by 10 min in the second equilibration buffer containing 2.5% iodoacetamide instead of DTT.

In the case of OFFGEL prefractionation, 24 fractions were combined into 8 samples, desalted, and vacuum-dried as described earlier. Each sample was dissolved in 125 μL of IPG buffer and centrifuged at 20000 rcf for 60 min at 10 °C before application to 7 cm ReadyStrip IPG Strips of the appropriate narrow pH range (Bio-Rad) by passive rehydration. Prior to the second dimension, the IPG strips were equilibrated as stated above. IEF was performed in the Protean IEF Cell (Bio-Rad) for 19000 Vh.

**SDS-PAGE (Second Dimension of 2-DE), Staining, and Image Analysis.** The second dimension was run on 20.0 × 20.5 cm or 8.3 × 6.4 cm 15%T vertical polyacrylamide gels in the Protean Plus Dodeca

Cell (Bio-Rad) or Mini-Protean 3 Dodeca Cell (Bio-Rad) for desalted beer samples or prefractionated samples, respectively. The Precision Plus Protein Standard (Bio-Rad) molecular weight standard was applied to each gel. Gels were stained with silver nitrate MS compatible protocol using the ProteoSilver Plus Silver Stain Kit (Sigma-Aldrich) or with Sypro Ruby (Invitrogen) fluorescent dye according to protocols recommended by the suppliers. Gels were scanned by calibrated densitometer GS-800 (Bio-Rad) or FLA-7000 Fluorescent Image Analyzer (Fujifilm) and processed by PDQuest 8.0.1 Advanced software (Bio-Rad). The Spot Detection Wizard was used to select the parameters for spot detection; a faint spot (the sensitivity and minimum peak value parameter) and a large spot cluster (the radius of the background subtraction) were selected. Gel warping was done prior to spot matching. Results of automated spot detection and matching were checked and manually corrected if necessary. For evaluation of the country of origin impact on the proteome, two new gel groups were created: local beers (A + C) and imported beers (B + D); the quantitative and qualitative analytical sets were used for comparison. Protein spots were considered as present if spot intensity was at least 10-fold higher than background and the corresponding spot was present on at least two of three gel replicates. Local regression model (LOESS) was used for spot intensity normalization. A 2-fold change in average spot intensity between compared gel groups was taken as significant.

**Mass Spectrometric Analysis.** Protein spots to be analyzed were excised manually or by means of the EXQuest spot cutter (Bio-Rad) from the corresponding 2-DE gels. After destaining and washing procedures, each gel plug was incubated with trypsin. MALDI-MS/MS analyses were performed on an Ultraflex III mass spectrometer (Bruker Daltonik). Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of beer tryptic peptides was performed using a EASY-nLC system (Proxeon) online coupled with an HCTultra PTM Discovery System ion trap mass spectrometer (Bruker Daltonik). The MASCOT 2.2 (MatrixScience) search engine was used for processing the MS and MS/MS data. Database searches were carried out in comparison with the nonredundant version of the NCBI database, NCBInr (version 110513, 14086771 sequences in total, 885409 sequences in Green Plants taxonomy; for details cf. the Supporting Information).

## RESULTS AND DISCUSSION

Beer represents a relatively complex mixture of compounds of different classes that orchestrate the final taste and quality. Unfortunately, several types of these compounds, mainly low molecular weight components such as polyphenols, can interfere with the successful analysis of beer proteins, especially in electrophoretic separation. Even reliable determination of total protein content is rather difficult in raw beer. Application of several common methods, that is, the Bradford assay[16] (Coomassie Brilliant Blue binding, CBB), the bicinchoninic acid assay (BCA), and the measurement of absorbance at 280 nm, have been reported for protein content determination in raw beer; however, the diverse methods yielded rather disparate responses.[17,18] Whereas BCA and absorbance at 280 nm suffered from strong interference from polyphenols, CBB responded to polyphenols only to a limited extent. Total protein in our analyzed samples of degassed raw beer measured by the Bradford assay showed average concentrations (mg/mL) in different lagers as follows: A, 0.44; B, 0.37; C, 0.33; D, 0.49. These results are in good agreement with values reported from Bradford assay measurements by Abernathy et al.[18]

Primarily we focused on selection of appropriate protein isolation method from raw beer samples, allowing the removal of interfering compounds. Several techniques of protein isolation based on different separation principles were compared. We tested two traditional precipitation protocols, acetone/trichloroacetic acid precipitation and ethanol precipitation, as well as a commercial precipitation kit declaring enhanced protein precipitation efficiency over traditional protocols. The precipitation techniques were compared with two variants of gel filtration techniques: Sephadex G-25 disposable columns and liquid chromatographic fractionation using HiTrap column. Aliquots of the same beer sample were processed according to the particular protocol, and protein recovery was measured according to the Bradford assay. As expected, all precipitation protocols led to significant protein loss (Table 1). Both gel filtration techniques gave comparable recoveries of beer proteins (about 60%), which contrasted with lower recoveries after the tested precipitation procedures. Protein separation patterns were confirmed by 2-DE. No preference for isolation of particular protein groups was observed (images not shown) within the selected isolation methods. On the basis of these results (Table 1) preparative gel chromatography on the HiTrap column (Sephadex G-25) was used as the standard protein isolation method in the subsequent experiments.

**Table 1. Protein Recoveries Depending on Protein Isolation Technique, Calculated as Ratio of Protein Amount Prior to and after the Protein Isolation Step[a]**

| method | recovery (%) |
|---|---|
| precipitation, acetone/TCA | 16 |
| precipitation kit[b] | 27 |
| precipitation, ethanol | 36 |
| Sephadex G-25, NAP[c] | 60 |
| Sephadex G-25, HiTrap | 62 |

[a]Dried raw beer as well as other dried samples (after precipitation or gel chromatography) were dissolved in IPG buffer, and the protein concentration was assayed according to the method of Bradford.[16] [b]ProteoExtract Protein Precipitation Kit (Calbiochem). [c]NAP-25 Column (GE Healthcare).

Proteins from two lager beers made in Czech Republic (A, Pilsner Urquell; C, Budweiser Budvar) and two made in other countries (B, Stella Artois, Belgium; D, Heineken, The Netherlands) were isolated by the selected procedure. Isolated beer proteins were initially separated by 2-DE. Three parallel gels were run for each lager sample and processed simultaneously in one Dodeca cell. We used the urea–thiourea containing IPG buffer for the first dimension, as it exhibits a superior solubilizing power and is capable of increasing protein solubility during the first dimension of the separation, as well as protein transfer to the second gel dimension.[19] The silver staining protocol used at the beginning of the project for visualization of protein spots was abandoned, because it was impossible to stain minor spots without overstaining the area close to abundant protein Z, which was in agreement with Perrocheau.[6] Their group had to compromise the silver staining sensitivity versus protein Z abundance, which limited the total number of proteins they could identify. Fluorescent Sypro Ruby staining, which retains sensitivity comparable to silver staining and preserves linearity throughout several orders of magnitude of protein concentration,[13] was employed for spot visualization. The average number of protein spots for individual lagers was about 130.

Protein spots selected by image analysis were excised and analyzed by MALDI-MS/MS and LC-MS/MS. Analyses of more than 300 protein spots led to the identification of 52 proteins, mostly from *H. vulgare* (20 proteins) and *Saccharomyces* species (25 proteins) (Table 2; Table S1 in the Supporting Information),

**Table 2. Summary of Qualitative and Quantitative Changes in Protein Spot Quantities Found When Protein Maps of Two Individual Lager Beers Were Compared[a]**

| | number of spots with significant change | | | | | | |
|---|---|---|---|---|---|---|---|
| | qualitative | | | quantitative | | | |
| comparison (1st vs 2nd) | 1st > 2nd | 1st < 2nd | subtotal | 1st > 2nd | 1st < 2nd | subtotal | total |
| A vs B | 0 | 13 | 13 | 1 | 8 | 9 | 22 |
| A vs C | 6 | 3 | 9 | 6 | 1 | 7 | 16 |
| A vs D | 1 | 25 | 26 | 6 | 6 | 12 | 38 |
| B vs C | 26 | 5 | 31 | 4 | 5 | 9 | 40 |
| B vs D | 5 | 9 | 14 | 11 | 5 | 16 | 30 |
| C vs D | 5 | 33 | 38 | 7 | 13 | 20 | 58 |

[a]Lager beers labels are as follows: A, Pilsner Urquell; B, Stella Artois; C, Budweiser Budvar; D, Heineken.

as most proteins were found in several spots, indicating the presence of different isoforms, modified forms, or fragments of proteins. The total number of proteins identified after 2-DE without any prefractionation seems to be the largest set reported up to now. Recently published data concerning protein identification in beer samples using MALDI-MS/MS or LC-MS/MS after 2-DE separation without any prefractionation yielded only about 15 proteins.[1,2,6] However, it has to be noted that this small number of identified proteins could be related to the release date and source of the reference protein database (e.g., NCBI or SwissProt) used during MS/MS searches as stressed, for example, by Perrocheau et al.[6] Although the databases are continuously upgraded, barley proteome information is still not complete. Despite the use of the most recent nonredundant NCBI database (NCBInr, version 110513) for searching our MS/MS data, several proteins were identified as wheat and rice proteins, which could be probably due to the absence of entries of the corresponding barley proteins. On the other hand, no detailed information concerning brewing ingredients was provided by the brewing companies.

Results of image analysis were used for comparison of the corresponding protein maps (Figures 1−3) with the aim of finding qualitative or quantitative differences among individual analyzed lagers related to their country of origin. The most prevalent difference between Czech lager beers and imported beers consisted in the gel region around the protein Z zone, where >10 protein spots were absent in the case of Czech lager

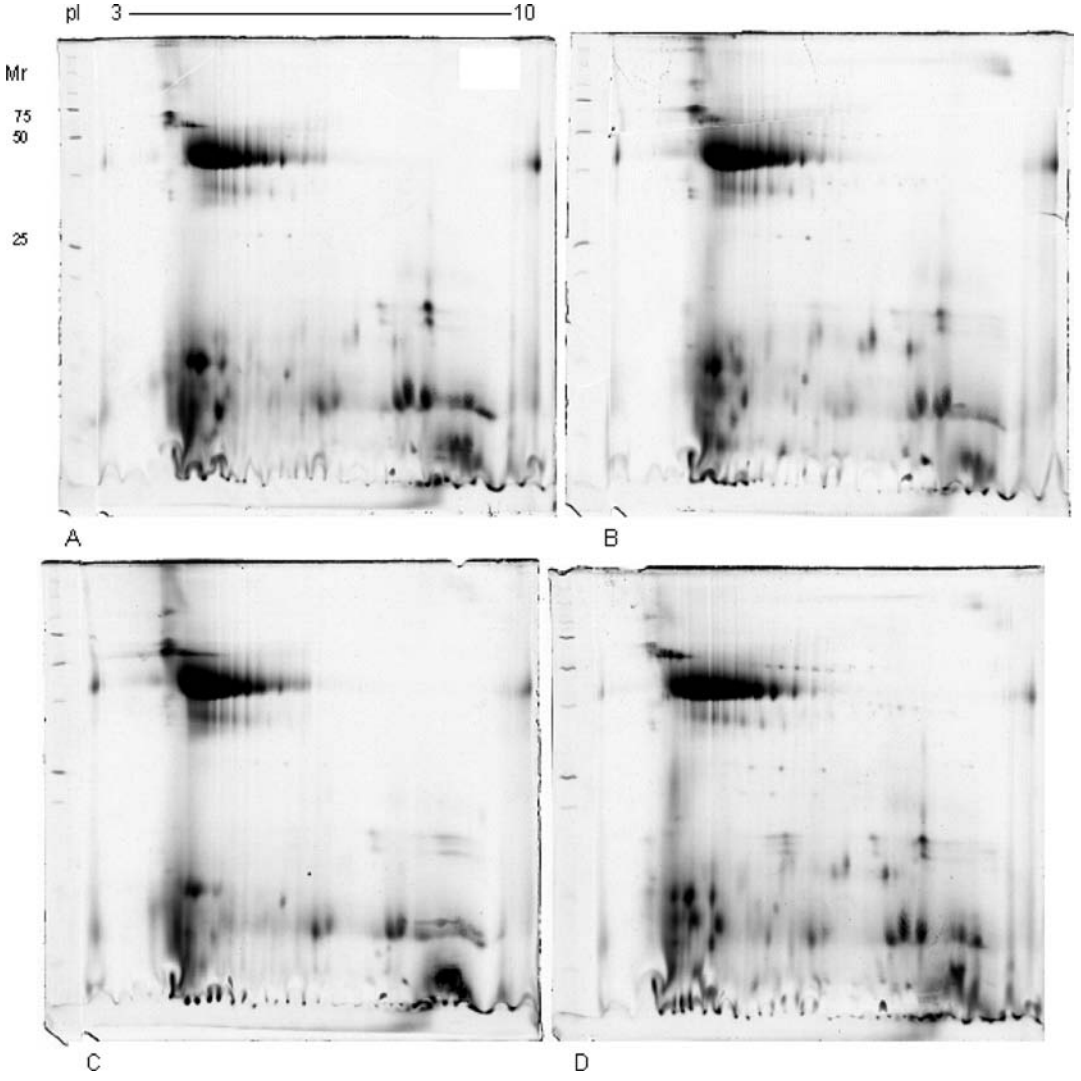


**Figure 1.** 2-DE patterns of four samples of beer without prefractionation. Lager beers labels are as follows: A, Pilsner Urquell; B, Stella Artois; C, Budweiser Budvar; D, Heineken. Beers A and C were made in Czech Republic; beers B and D were made in other countries. First dimension: 18 cm long IPG strips (pH range 3−10NL); 2-DE gels were stained by Sypro Ruby.

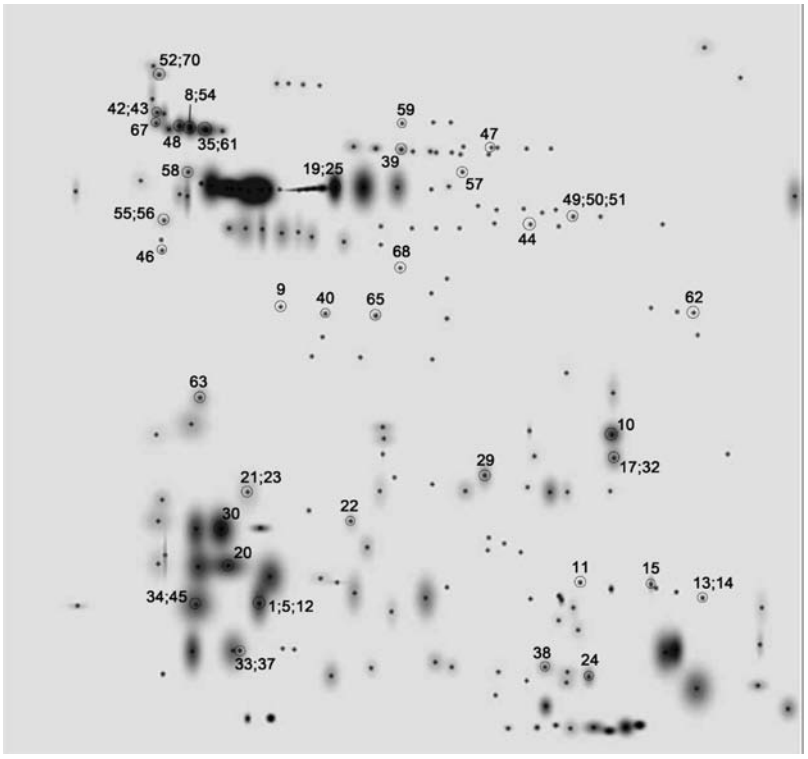


**Figure 2.** Master gel (created in PDQuest software) representing all spots found in samples A, B, C, and D. Assigned numbers indicate proteins with the most significant (highest protein score) identification (see Table 3).

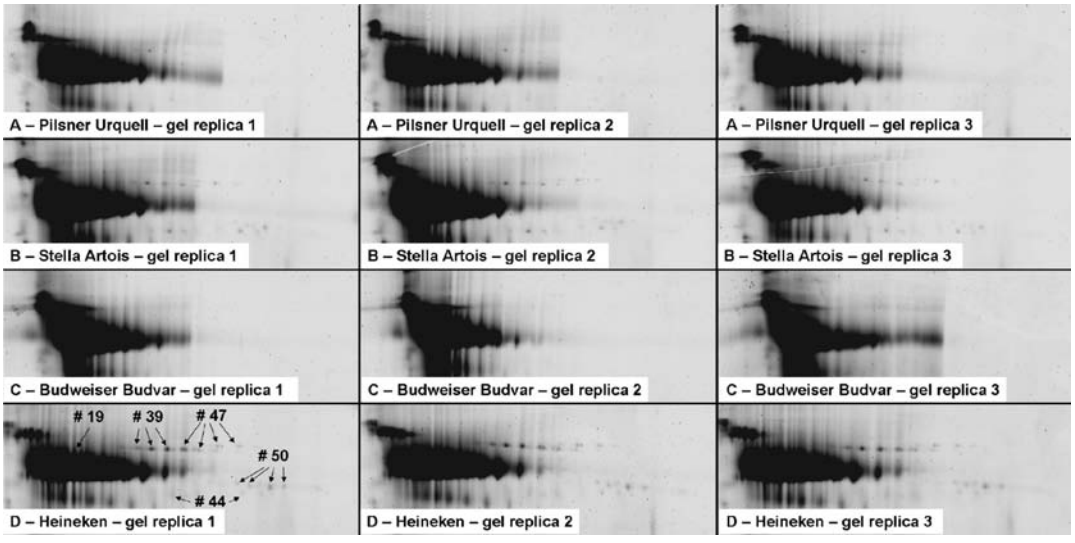


**Figure 3.** Selected qualitative differences between Czech (A, C) and imported (B, D) lager beers observed near the protein Z (19) zone. Proteins identified in respective spots are shown in Table 3 (or cf. Supporting Information tables for details). Sypro Ruby was used for protein visualization.

beers (Figure 3). MS/MS analysis revealed mainly the presence of proteins from *Saccharomyces* species (e.g., two enolase isoforms, proteins 39 and 47; glyceraldehyde 3-phosphate dehydrogenase, protein 50; coproporphyrinogen III oxidase, protein 44). Results indicate that analyzed Czech lager beers and imported beers differ qualitatively mainly in these proteins originating from *Saccharomyces* species. This phenomenon could be related to different yeast cultures used during brewing technology or the processing technology itself. Besides qualitative differences, 14 quantitatively different protein spots were also found. Imported beers showed higher spot intensities as compared to Czech beers in seven cases, whereas the same number of spots was of lower intensity. All 14 spots were subjected to protein identification. Unfortunately, all spots contained more than one protein, and no conclusions could be drawn with respect to the observed quantitative differences. Proteins identified included, for example, protein Z (or its fragments), LTP proteins, trypsin/amylase inhibitors, and a few yeast proteins. All identified proteins were also observed in other protein spots indicating that the observed differences possibly resulted from the presence of protein isoforms (or differently modified forms).

Differences among individual lager beers were studied as well. Image analysis of protein maps of individual lager beers

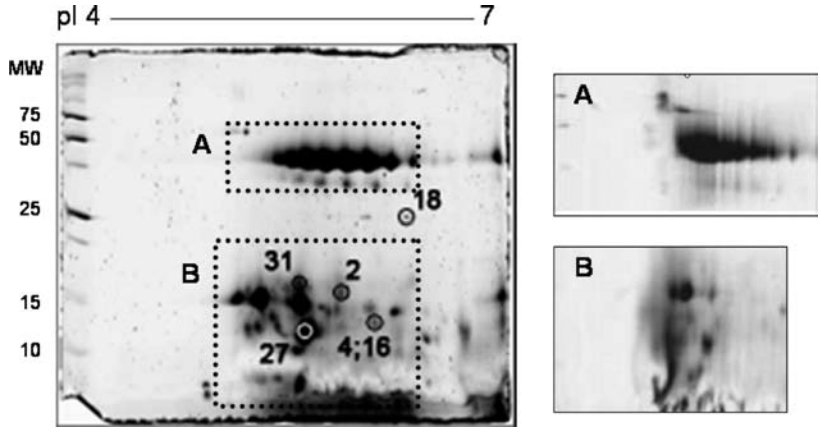


**Figure 4.** (Left) OFFGEL beer fraction IV (7 cm strip, pH 4–7, 220 μg of protein). Assigned numbers indicate identified proteins listed in Table 3 (boldfaced entries). (Right) Unfractionated proteins (as in Figure 1). Large gel sections (18 cm strip, pH 3–10NL) correspond with dotted-line areas. Both gels were Sypro Ruby stained.

was performed to ascertain qualitative and quantitative differences; all possible combinations resulted in six comparisons (Table 2). More than 200 differences were observed in total. Certain spots were found as different in more than one comparison (5 spots were found different 4 times; 25 spots, 3 times; and 40 spots, 2 times), leading to 99 protein spots responsible for all observed changes. Lager beer D showed the highest number of protein spots with higher intensities than intensities of corresponding spots of all three other tested lagers (91 spots), whereas lager beer C showed the lowest one (20 spots).

To improve separation of protein isolates the prefractionation step prior to 2-DE was applied. Two prefractionation procedures were tested: preparative IEF fractionation in solution (OFFGEL) and commercially available ProteoMiner Protein Enrichment Kit (Bio-Rad). In the case of ProteoMiner, both TCA/acetone precipitated beer and raw beer were used as samples processed with the Proteominer kit, followed by 2-DE using 7 cm IPG strips. However, the results (data not shown) were not satisfactory in terms of reduction of protein Z abundance, most likely because the peptide library in the kit is dedicated to blood plasma protein analysis. Our findings are in agreement with the recently published study of Fasoli et al.[15] They achieved successful enrichment of the low-abundance proteins with simultaneous minimal less abundant components in the capture of high-abundance species in beer only after the application of a new homemade combinatorial peptide ligand library.

The second tested prefractionation approach, preparative OFFGEL prefractionation, allowed the application of a preparative initial amount (5 mg) of total protein per OFFGEL strip, compared to 0.6 mg used originally for 2-DE. This high amount facilitated protein identification in complex protein samples. Volumes of each three sequential wells were pooled because the protein concentration in each fraction was not optimal for direct 2-DE separation and detection.

To ensure the identical solubilization conditions for all experiments (including the original nonprefractionated samples), all of the pooled fractions were desalted, dried under vacuum, and dissolved in IPG buffer. The distribution of proteins in particular pooled fractions was checked beforehand to assign a proper range of each IPG strip: 63 μL of each pooled fraction was applied on the 2-DE gel using a 7 cm long IPG strip (data not shown). On the basis of this separation the pooled fractions were applied onto eight 7 cm strips of appropriate pH range. Prefractionation of proteins according to their p*I* was beneficial mainly in combination with IEF on narrow-range IPG strips (Figure 4). The prefractionation enabled elimination of the most abundant protein Z from several fractions (especially from pooled fractions I, II, and V–VIII). Detection of minor protein spots was thus enabled in these fractions. Newly observed protein spots were subjected to identification that led to enlargement of the spectrum of observed beer proteins by 18 additional entries (Table 3; for further details regarding the protein identification cf. Table S1 in the Supporting Information). Analyses of other selected protein spots from 2-DE gels of particular fractions led to confirmation of already identified proteins.

Because the improved protein separation is necessary for better characterization of proteome components, the OFFGEL prefractionation step represents one of the promising strategies for improving 2-DE resolution power in beer proteome analysis. Different protein Z isoforms could be separated in the narrow-range strips, which can enable further studies of its posttranslational modifications, especially glycosylations, which are very common in beer due to a variety of factors.[20,21] At present, gel-based glycoprotein analysis is still one of the main experimental strategies providing the most information on individual plant glycoproteins.[22]

In total, 70 beer proteins were identified in our experiments. Most of the identified proteins originated from *H. vulgare* (30 proteins), including the most abundant protein Z, groups of LTP proteins, and trypsin inhibitors. Hordeins potentially triggering allergic response[8] were found in all beers. The rest of the proteins originated mostly from *Saccharomyces* species (31 proteins). As already mentioned above, proteins from rice (*Oryza sativa*, 4 proteins) and wheat (*Triticum aestivum*, 4 proteins) were observed as well. This phenomenon is probably related to the not completely sequenced barley genome and partial sequence homology of barley, rice, and wheat proteins.

Fasoli et al.[15] reported comparable protein numbers (63 proteins, 20 from *H. vulgare* and 42 from *Saccharomyces* species) when they used a new homemade combinatorial peptide ligand library (CPLL) in combination with 1-DE and tandem mass spectrometry. The difference in sample preparation/separation strategy induced our interest in the overlap of proteins identified in our study and that of Fasoli et al. Due to the inconsistency between both studies (different brands of beer,

**Table 3. Summary of All Proteins Identified in Four Studied Lager Beers[a]**

| no. | GI no. | protein name | score | pept | no. | GI no. | protein name | score | pept |
|---|---|---|---|---|---|---|---|---|---|
| | *Hordeum vulgare* | | | | | *Saccharomyces* species | | | |
| 1[b] | gil509070 | 18 $K_d$ heat shock protein | 216 | 1 | 39 | gil6321968 | 2-phosphoglycerate dehydratase | 1583 | 13 |
| **2[b]** | **gil18955** | **α-amylase inhibitor** | **218** | **1** | 40 | gil10383781 | 3-phosphoglycerate kinase | 838 | 10 |
| **3** | **gil169666634** | **calcium-dependent protein kinase** | **531** | **5** | **41** | **gil48428723** | **acyl-CoA-binding protein 2** | **72** | **3** |
| **4[b]** | **gil109238647** | **cystatin Hv-CPI6** | **61** | **1** | 42 | gil6323964 | cell wall protein, Scw10p | 644 | 8 |
| 5 | gil671537 | D-hordein | 265 | 5 | 43 | gil6321718 | cell wall protein, Scw4p | 298 | 7 |
| **6[b]** | **gil226755** | **γ- hordein** | **106** | **2** | 44 | gil6320249 | coproporphyrinogen III oxidase | 105 | 5 |
| **7[b]** | **gil7431022** | **glucose and ribitol dehydrogenase homologue - barley** | **60** | **1** | 45 | gil6321648 | cytoplasmic thioredoxin isoenzyme | 369 | 7 |
| 8[b] | gil54661662 | grain softness protein | 135 | 2 | 46 | gil6321721 | endo-β-1,3-glucanase | 797 | 7 |
| 9[b] | gil224385 | hordein B | 246 | 1 | 47 | gil171455 | enolase | 1542 | 15 |
| 10[b] | gil54661047 | hordoindoline-b1 | 112 | 1 | 48[b] | gil46395590 | glucan 1,3-β-glucosidase | 90 | 1 |
| 11 | gil47168353 | lipid transfer protein 1 | 669 | 6 | 49[b] | gil219564313 | glyceraldehyde 3-phosphate dehydrogenase | 1111 | 13 |
| 12[b] | gil19005 | chymotrypsin inhibitor 2 | 111 | 1 | 50 | gil219564301 | glyceraldehyde 3-phosphate dehydrogenase | 1190 | 14 |
| 13[b] | gil6492243 | lipid transfer protein | 57 | 1 | 51 | gil6322409 | glyceraldehyde-3-phosphate dehydrogenase, isozyme 1 | 708 | 8 |
| 14[b] | gil1808651 | pathogenesis-related protein 4 | 215 | 1 | 52 | gil3730 | glycolipid-anchored surface protein | 195 | 8 |
| 15 | gil326491097 | predicted protein | 108 | 2 | **53** | **gil349747** | **heat shock protein of HSP70 family** | **88** | **2** |
| **16[b]** | **gil326494858** | **predicted protein** | **113** | **1** | 54 | gil6323331 | major exo-1,3-β-glucanase | 491 | 9 |
| 17[b] | gil326501830 | predicted protein | 248 | 2 | 55 | gil6322303 | mannose-containing glycoprotein | 75 | 4 |
| **18[b]** | **gil326503316** | **predicted protein** | **75** | **1** | 56 | gil968906 | NCA3 | 299 | 5 |
| 19 | gil1310677 | protein z-type serpin | 6082 | 17 | 57[b] | gil6321973 | Oye2p | 82 | 1 |
| 20 | gil123970 | α-amylase inhibitor BDAI-1 | 716 | 6 | 58 | gil6325103 | Pep4p | 80 | 4 |
| 21 | gil2506771 | α-amylase inhibitor BMAI-1 | 130 | 3 | 59 | gil6319673 | Pgi1p | 446 | 4 |
| 22 | gil585290 | α-amylase/trypsin inhibitor CMb | 108 | 6 | **60[b]** | **gil6324696** | **profilin** | **86** | **4** |
| 23 | gil585291 | α-amylase/trypsin inhibitor CMd | 237 | 3 | 61[b] | gil6322382 | Pry1p | 70 | 1 |
| 24 | gil128377 | probable nonspecific lipid-transfer protein | 573 | 2 | 62 | gil6322697 | tetrameric phosphoglycerate mutase | 519 | 9 |
| 25 | gil75282567 | serpin-Z7 | 267 | 9 | 63 | gil6323138 | thioredoxin peroxidase | 757 | 4 |
| **26[b]** | **gil75281963** | **serpin-ZX** | **115** | **3** | **64[b]** | **gil6319638** | **Tos1p** | **132** | **1** |
| **27** | **gil124122** | **subtilisin−chymotrypsin inhibitor 2A** | **134** | **3** | 65 | gil6320255 | triose phosphate isomerase, abundant glycolytic enzyme | 755 | 7 |
| **28[b]** | **gil2507469** | **triosephosphate isomerase, cytosolic** | **66** | **1** | **66** | **gil731388** | **uncharacterized protein YER188W** | **227** | **2** |
| 29 | gil1405736 | trypsin inhibitor cme precursor | 300 | 4 | 67[b] | gil486485 | UTH1 | 126 | 3 |
| 30 | gil225102 | trypsin/amylase inhibitor pUP13 | 913 | 5 | 68 | gil6320775 | vacuolar proteinase B | 211 | 6 |
| | | | | | **69[b]** | **gil6321179** | **Vel1p** | **82** | **3** |
| | *Triticum aestivum* | | | | | ***Aspergillus niger*** | | | |
| **31[b]** | **gil21711** | **CM 17 protein precursor** | **67** | **2** | 70 | gil224027 | glucoamylase G1 | 469 | 7 |
| 32 | gil281335542 | gliadin/avenin-like seed protein | 255 | 2 | | | | | |
| 33 | gil215398472 | globulin 3B | 111 | 2 | | | | | |
| 34[b] | gil32328625 | high molecular weight glutenin subunit | 586 | 2 | | | | | |
| | *Oryza sativa* | | | | | | | | |
| 35[b] | gil297720697 | Os01g0915900 | 91 | 1 | | | | | |
| **36[b]** | **gil115453373** | **Os03g0393900** | **61** | **1** | | | | | |
| 37 | gil297606280 | Os06g0650100 | 115 | 3 | | | | | |
| 38 | gil149391359 | polyubiquitin containing 7 ubiquitin monomers | 114 | 6 | | | | | |

[a]Proteins identified only after OFFGEL prefractionation are in boldface. Protein scores (score) and number of peptides (Pept) are shown. [b]Protein identification by one peptide with significant score.

different analytical and data processing procedures), the comparison of both protein sets were performed. A reference protein database consisting of all proteins identified by Fasoli et al. was created. Blast search for all proteins identified in our study (Table 3 and Table S1 of the Supporting Information) was done against the reference protein database. The respective protein was accepted as unique to our study (as compared to Fasoli et al.) when no common tryptic peptide was found between a particular protein and the reference protein database (single amino acid exchange was tolerated; for details, see Table S2 of the Supporting Information). The comparison revealed relatively small overlap in the identified proteins concerning 33 proteins (Figure 5; Table S2 of the Supporting Information).

Subsequent comparison indicated that the relatively small overlap of our protein set with that of Fasoli et al. is most probably

**Table 4. Number of Beer Proteins Originating from *Hordeum vulgare* (HV), *Triticum aestivum* (TA), *Oryza sativa* (OS), *Zea mays* (ZM), *Saccharomyces* Species (S), and Other Organisms Identified by Different Sources**

| source | separation | gel staining | HV | TA | OS | ZM | S | other | total |
|---|---|---|---|---|---|---|---|---|---|
| Perrocheau et al. | 2D | silver | 9 | | 1 | | 2 | | 12 |
| Fasoli et al. | CPLL[a] + 1D | Coomassie | 20 | | | 2 | 40 | 2 | 64 |
| this study | 2D | Sypro Ruby | 20 | 3 | 3 | | 25 | 1 | 52 |
| this study | OFFGEL + 2D | Sypro Ruby | 30 | 4 | 4 | | 31 | 1 | 70 |

[a]Proteominer-like prefractionation approach utilizing combinatorial peptide ligand libraries (CPLL) used for prefractionation prior to beer sample application onto 1D gel. Homemade CPLL was used.

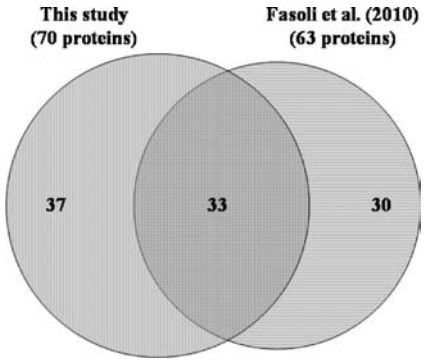


**Figure 5.** Number of proteins found in this study and in Fasoli et al.[15]

caused mainly by principal differences in the nature of employed sample preparation procedures (not by use of different protein databases and data processing; SwissProt database/Sequest in Fasoli et al. versus NCBInr database/Mascot in this study). First, we found more than 20 proteins of 37 proteins observed only in our study to be identifiable also using the SwissProt database (see the Supporting Information for more details). Second, we were not able to identify 30 proteins from the protein set reported by Fasoli et al. (despite the fact that the NCBInr database contains the corresponding protein entries with identical sequences).

Preparative gel filtration chromatography of beer was successfully applied for the removal of interfering compounds prior to two-dimensional gel electrophoresis of beer. The fluorescent staining was used to reach the high sensitivity, which at the same time did not cause the heavy overstaining close to protein Z, and supports the possibility of quantification as well. In addition, OFFGEL fractionation facilitated identification of proteins not observed in beer before, and, by use of IEF on narrow range strips, also allowed separation of several protein isoforms, which can enable their detailed study.

In combination with the OFFGEL prefractionation step, a total of 70 individual proteins were identified in examined beers (Table 4). Identified proteins originated from *H. vulgare* (30 proteins), from *Saccharomyces* species (31 proteins), and from other sources (9 proteins). Despite improved performance of the protocol used in this study, there is not enough information yet to come to a conclusion as to which protein markers could be the best candidates for inspecting the origin of beer in relation with trademark protection. The comparison of identified proteins revealed relatively small overlap with the protein set reported by Fasoli et al. This fact indicates the significance of sample preparation procedure selection and the necessity of simultaneous employing alternative protocols for increasing the integrity of information obtained from the sample.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Funding

This work was supported by the project "CEITEC − Central European Institute of Technology" (CZ.1.05/1.1.00/02.0068) from the European Regional Development Fund, by the Czech Ministry of Education, Youth and Sports (projects LC06034, 2B08057, and CZ.1.05/2.1.00/01.0012), and by the Czech Ministry of Agriculture (project QI91A069).

### Notes

The authors declare no competing financial interest.

## ■ REFERENCES

(1) Iimure, T.; Nankaku, N.; Hirota, N.; Tiansu, Z.; Hoki, T.; Kihara, M.; Hayashi, K.; Ito, K.; Sato, K. Construction of a novel beer proteome map and its use in beer quality control. *Food Chem.* **2010**, *118*, 566−574.

(2) Iimure, T.; Nankaku, N.; Watanabe-Sugimoto, M.; Hirota, N.; Tiansu, Z.; Kihara, M.; et al. Identification of novel haze-active beer proteins by proteome analysis. *J. Cereal Sci.* **2009**, *49*, 141−147.

(3) Okada, Y.; Iimure, T.; Takoi, K.; Kaneko, T.; Kihara, M.; Hayashi, K.; et al. The influence of barley malt protein modification on beer foam stability and their relationship to the barley dimeric α-amylase inhibitor-I (BDAI-I) as a possible foam-promoting protein. *J. Agric. Food Chem.* **2008**, *56*, 1458−1464.

(4) Iimure, T.; Takoi, K.; Kaneko, T.; Kihara, M.; Hayashi, K.; Ito, K.; et al. Novel prediction method of beer foam stability using protein Z, barley dimeric amylase inhibitor-1 (BDAI-1) and yeast thioredoxin. *J. Agric. Food Chem.* **2008**, *56*, 8664−8671.

(5) Hao, J.; Li, Q.; Dong, J.; Yu, J.; Gu, G.; Fan, W.; Chen, J. Identification of the major proteins in beer foam by mass spectrometry following sodium dodecyl sulfate−polyacrylamide gel electrophoresis. *J. Am. Soc. Brew. Chem.* **2006**, *64*, 166−174.

(6) Perrocheau, L.; Rogniaux, H.; Boivin, P.; Marion, D. Probing heat-stable water-soluble proteins from barley to malt and beer. *Proteomics* **2005**, *5*, 2849−2858.

(7) Čížková, H.; Dostálek, P.; Fiala, J.; Kolouchová, I. Importance of proteins from the viewpoint of stability of the beer foam. *Chem. Listy* **2006**, *100*, 478−485.

(8) Picariello, G.; Bonomi, F.; Iametti, S.; Rasmussen, P.; Pepe, C.; Lilla, S.; Ferranti, P. Proteomic and peptidomic characterisation of beer: Immunological and technological implications. *Food Chem.* **2011**, *124*, 1718−1726.

(9) Perrocheau, L.; Bakan, B.; Boivin, P.; Marion, D. Stability of barley and malt lipid transfer protein 1 (LTP1) toward heating and reducing agents: relationships with the brewing process. *J. Agric. Food Chem.* **2006**, *54*, 3108−3113.

(10) Klose, Ch.; Schehl, B. D.; Arendt, E. K. Protein changes during malting of Barley using novel lab-on-a-chip technology in comparison to two-dimensional gel electrophoresis. *Brew. Sci.* **2008**, *61*, 56−65.

(11) Robinson, L. H.; Healy, P.; Stewart, D. C.; Eglinton, J. K.; Ford, Ch. M.; Evans, D. E. The identification of a barley haze active protein that influences beer haze stability: the genetic basis of a barley malt haze active protein. *J. Cereal Sci.* **2007**, *45*, 335−342.
(12) Gorinstein, S.; Zemser, M.; Vargas-Albores, F.; Ochoa, J.-L.; Paredes-Lopez, O.; Scheler, Ch.; Salnikow, J.; Martin-Belloso, O.; Trakhtenberg, S. Proteins and amino acids in beers, their contents and relationships with other analytical data. *Food Chem.* **1999**, *67*, 71−78.
(13) Rabilloud, T.; Chevallet, M.; Luche, S.; Lelong, C. Two-dimensional gel electrophoresis in proteomics: past, present and future. *J. Proteomics* **2010**, *73*, 2064−2077.
(14) Righetti, P. G.; Castagna, A.; Herbert, B.; Reymond, F.; Rossier, J. S. Prefractionation techniques in proteome analysis. *Proteomics* **2003**, *3*, 1397−1407.
(15) Fasoli, E.; Aldini, G.; Regazzoni, L.; Kravchuk, A. V.; Citterio, A.; Righetti, P. G. Les Maîtres de l'Orge: the proteome content of your beer mug. *J. Proteome Res.* **2010**, *9*, 5262−5269.
(16) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(17) Siebert, K. J.; Lynn, P. Y. Comparison of methods for measuring protein in beer. *J. Am. Soc. Brew. Chem.* **2006**, *63*, 163−170.
(18) Abernathy, D.; Spedding, G.; Starcher, B. Analysis of protein and total usable nitrogen in beer and wine using a microwell ninhydrin assay. *J. Inst. Brew.* **2009**, *2*, 122−127.
(19) Rabilloud, T. Solubilization of proteins in 2D electrophoresis: an outline. In *Methods in Molecular Biology. Two-Dimensional Electrophoresis Protocols*; Sheehan, D., Tyther, R., Eds.; Humana Press: Totowa, NJ, 2009; Vol. *519*, pp 19−30.
(20) Jegou, S.; Douliez, J.-P.; Molle, D.; Boivin, P.; Marion, D. Purification and structural characterization of LTP1 polypeptides from beer. *J. Agric. Food Chem.* **2000**, *48*, 5023−5029.
(21) Wu, M. J.; Clarke, F. M.; Rogers, P. J.; Young, P.; Sales, N.; O'Doherty, P. J.; Higgins, V. J. Identification of a protein with antioxidant activity that is important for the protection against beer ageing. *Int. J. Mol. Sci.* **2011**, *12*, 6089−6103.
(22) Song, W.; Henquet, M. G. L.; Mentink, R. A.; van Dijk, A. J.; Cordewener, J. H. G.; Bosch, D.; America, A. H. P.; van der Krol, A. R. *N*-glycoproteomics in plants: perspectives and challenges. *J. Proteomics* **2011**, *74*, 1464−1474.