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Protein Changes during Malting of Barley using Novel Lab-on-a-Chip Technology in Comparison to two-dimensional Gel Electrophoresis

During the malting process storage proteins are degraded by proteolytic enzymes into small peptides and amino acids. The activity of these enzymes was measured at various stages during malting of barley and was found to be increased. To visualize proteolytic degradation, proteins of unmalted, germinating and malted grains were fractionated. After extracting the proteins on the basis of their solubility (Osborne fractionation) protein fractions were analysed using a Lab-on-a-Chip technique, which separates the proteins, based on their molecular weight, by capillary electrophoresis. This new technique for the analysis of proteins was supported by using two-dimensional gel electrophoresis. In addition, amino acid analysis on barley and barley malt was carried out. In general a degradation of the proteins to small peptides and amino acids could be observed in all fractions. In the albumin and globulin fraction also a protein increase was observed, which is due to the fact that these fractions contain the majority of the metabolically active proteins. The Lab-on-a-Chip analysis technique was found to be appropriate for analysis of degrading or increasing proteins, as it revealed rapid, repeatable and reliable results, which could be validated by using common protein analysis techniques such as two-dimensional gel electrophoresis and amino acid analysis.

Descriptors: barley, Lab-on-a-Chip analysis, malting, protein changes, protein fractions, two-dimensional gel electrophoresis

1 Introduction

Barley (*Hordeum vulgare*) has been traditionally used for the production of malt, which is applied in commercial brewing. The protein content of barley varies between 8 and 13 % [1] of which about a third passes into the final beer. According to their solubility proteins can be divided into different fractions. Albumins are soluble in water and account for only 3–5 % of the total protein. Most of the information found in the literature about albumins reveals two main proteins, protein Z, which is a glycoprotein with the molecular weight of ~40 kDa and lipid transfer protein 1 (LTP1), which is a 9.7 kDa glycoprotein with 91 amino acid residues. Both proteins are also present in beer and have been related to foam formation, -stability and beer haze [2, 3, 4]. Globulins are soluble in saltwater and their quantity ranges from 10 to 20 % of the total protein. They consist of both, metabolically active proteins and storage proteins [1]. Storage proteins of the globulin fraction are located in the aleurone layer and the embryo and show similarities to the 7S vicilins of legumes [5]. Albumin and globulin fractions are also called soluble or metabolically active proteins, since they represent most of the enzymes. Alcohol soluble proteins are named prolamins or particularly in barley hordeins. Together with the residue protein fraction (glutelins) they form most of the barley storage proteins. The prolamin fraction is the most studied fraction of barley; they account for up to 50 % of the total protein content of the grain and function as storage proteins [5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15]. According to their electrophoretic mobility they have been classified into B-, C-, D- and γ-hordeins [16], whereas the B-hordeins account for 70–80 %, the C-hordeins for 10–20 % and the D- and γ-hordeins form less than 5 % of the total hordein fraction. Another classification of hordein proteins can be made based on their amino acid composition and sequence, where three groups can be defined, S (sulphur)-rich, S (sulphur)-poor and HMW proteins [15]. B-hordeins are sulphur rich and can be subdivided into B1, B2 and B3 subgroups [17].

Malting is the initial step in beer production and strongly defines type and quality of the beer. The main purpose of malting is to produce enzymes and to breakdown cell walls surrounding starch granules. One of the most important physical-chemical changes that occurs during malting is the degradation of the proteinaceous matrix that surrounds the starch granules within the cells of the endosperm and their conversion into soluble peptides and amino acids to provide substrates for the synthesis of proteins in

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Tables and figures see Appendix

the growing embryo [18]. Malt proteins have a high impact on the brewing process and the subsequent quality of beer. Protein content and size distribution are of particular interest in terms of filtering, fermentability, foam and haze stability. Thus, degradation and formation of proteins already during the malting process is essential for the quality of the final beer.

The aim of this study was to evaluate the protein changes from the raw barley over the germination to the final malt. For this purpose a novel analysis technique, the Lab-on-a-Chip capillary electrophoresis, was used and results were compared to results of two-dimensional gel electrophoresis.

2 Experimental

2.1 Materials and malting

The two rowed spring barley variety Prestige was harvested in 2005 and obtained from Cork Malting Company, Ireland. Malting was carried out using a micro malting machine (Joe White Malting Systems, Perth, Australia). For this purpose, 8 kg of barley seeds were soaked three times at 13 ˚C, followed by and an air rest stage at 18 ˚C. These two stages were carried out three times. After 31 h of steeping, seeds were germinated for 5 days and 6 h at three different temperatures (17 ˚C, 15 ˚C and 13 ˚C) and kilned at six increasing temperatures for 23 h using a schedule that started at 55 ˚C and increased to 60 ˚C, to 68 ˚C, to 72 ˚C, to 78 ˚C and finally to 85 ˚C. Unmalted, three days germinating and kilned samples were taken, freeze dried (VirTus Benchtop 4k, Biopharma Process Systems, Winchester, UK) and milled with a laboratory-scale disc mill (Bühler GmbH, Braunschweig, Germany) set at a fine setting of 0.05 mm and stored at –80 ˚C.

2.2 Total protein analysis of barley

The proteolytic enzyme activity during the malting process was measured according to the method of Brijs et al. [19] and was carried out once to show the tendency of proteolytic activity. The free amino acid profile of the milled samples was measured using a Jeol JLC-500/V amino acid analyser (Jeol (UK] Ltd., Garden city, Herts, UK) fitted with a Jeol Na^+ high performance cation exchange column. For the total amino acid profile, milled samples as well as each protein fraction were analysed according to the method of Moore and Stein [20]. Amino acid analysis was performed once to confirm existing values of amino acids found in barley.

2.3 Modified Osborne fractionation of barley proteins

Unmalted, germinating and malted barley (10 g of each sample) were defatted with 100 mL hexane using a soxhlet extractor. The defatted samples were then extracted twice with 100 mL of distilled water (albumin fraction). After water extraction the residue was extracted twice with 100 mL of 0.5 % NaCl (globulin fraction). The remaining flour was then extracted three times with 150 ml of 55 % 1-propanol + 1 % Dithiothreitol (DTT) (prolamin fraction) and the glutelin fraction together with the residue was extracted three times with a solvent containing 6M urea, 2 % sodium dodecyl sulfate (SDS) and 1 % DTT. Each extraction was carried out at room temperature for 10 min and centrifuged afterwards at 10,000 rpm for 10 min. All supernatants were collected, further dialyzed against distilled water for 24 h, freeze-dried and stored at –80 °C.

2.4 Lab-on-a-Chip analysis of extracted protein fractions

10 mg of each extracted protein fraction were dissolved in 1 mL of their extraction solvents and applied to the Agilent 2100 Bioanalyzer. 4 µL of solution were denatured using 2 µL of Agilent denaturing solution and heated for 5 min at 100 °C. After dilution with deionised water, 6 µL were applied to the Protein 230^+ LabChip and the Protein 80^+ LabChip for analysis in the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). For the 230^+ LabChip, each run included a ladder comprising reference proteins of 7, 15, 28, 46, 63, 95, 150 kDa plus an upper marker of 240 kDa and a lower marker of 4.5 kDa. Each sample contained an internal standard comprising the upper and lower marker as well. Any peak detected below 14 kDa is termed a system peak and is not included in analysis. The detection performance was also carried out in a molecular weight range between 4.5 and 95 kDa using the Protein 80^+ LabChip. For this analysis the ladder consisted of reference proteins of 3.5, 6.5, 15, 28, 46, 63 kDa plus the upper and the lower markers of 95 and 1.6 kDa. According to the Agilent manual any peak detected below 5 kDa is named a system peak and is not included in analysis. Results can be shown in an electropherogram or a gel-like image, as known from SDS-PAGE analysis, where the intensity of bands equals the peak heights in the electropherogram.

2.5 Two-dimensional gel electrophoresis of extracted protein fractions

Barley protein fractions of unmalted, germinating and malted samples were defrosted and two-dimensional gel electrophoresis was performed. All samples were dissolved in a solubilisation buffer containing 9M urea, 4 % CHAPS, 0.05 % Triton X100 and 65mM DTT. 125 µl of protein solution were applied to each strip. Isoelectrofocusing (IEF) was carried out using 7cm IPG 3–10 strips (ReadyStrip™, BioRad) and a Bio-Rad PROTEAN IEF cell with controlled cell temperature of 20 °C. The running conditions were as follows: Passive rehydration: 8 h, active rehydration (50 V): 8 h, rapid 300 V: 30 min, linear 4000 V: 20000 V-h, rapid 300 V: 99 h. After IEF was completed, the IPG strips were equilibrated for 15 min in a buffer containing 50 mM TrisHCl pH 8.8, 6 M urea, 30 % glycerol, 2 % SDS and130 mM DTT. After that the strips were equilibrated for another 15 min in the same solution except DTT was replaced with 130 mM iodoacetamide and traces of bromophenol blue. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a BioRad Criterion Dodeca cell with gels of total acrylamide concentration of 12.5 % at 20 °C, sealed with Tris/glycine/SDS running buffer. The gels were run at 100 V until the tracking dye reached the bottom of the gel. To visualize the proteins, the gels were first incubated in a fixation solution, containing ethanol and phosphoric acid and then in an incubation solution containing methanol, phosphoric acid and ammonium sulphate dissolved in distilled water. The staining was carried out with the incubation solution containing Coomassie brilliant blue for 4 days.

3 Results

3.1 Proteolytic activity

Total proteolytic enzyme activity was analysed using haemoglobin as substrate and absorption was measured against L-Leucine as standard. During the malting process a 6.2-fold increase in proteolytic activity could be observed. As shown in Figure 1, the results reveal an increase in the proteolytic activity level during the 31 h steeping stage (from 3.46 to 5.63 mg of L-leucine h^{-1} g^{-1}). After the first day of germination a large increase from 6.07 to 21.21 mg of leucine h^{-1} g^{-1} could be observed and during the last step of malting the proteolytic activity increases only slightly from 21.21 to 21.39 mg of leucine h^{-1} g^{-1}.

3.2 Protein fractions

To give a fundamental understanding, how proteins are affected by the increasing proteolytic acticity during malting, the four protein fractions of barley albumins, globulins, hordeins and glutelins were analysed.

3.2.1 Albumin fraction

The electropherogram of the water soluble fraction of unmalted, germinating and malted samples using the Protein 80^+ LabChip is shown in Figure 2A. Several protein peaks and peak areas could be detected. The largest area (1) ranges from 9.4 to 18.1 kDa and was decreased during the first three days of germination by 36.61 % and after kilning by 58.10 %. A smaller peak area (82) with the molecular weight of 22.8–27.2 kDa decreased slightly by 15.58 % first and by 35.51 % during the entire process. Another area of peaks (3) between the molecular weight of 34.9 and 40.3 kDa increased slightly during germination and decreased after kilning by 33.43 %. One protein with the size of 49.8 kDa (4) was detected with 6.4 Fluorescence Units (FU) only and raised sharply during germination to 13.7 FU and to 26.2 FU after kilning which equals a 3.09-fold increase. The protein with the size of 56 kDa (5) increased markedly during germination from 30.6 FU to 189.8 FU and was degraded again after kilning to 121.9 FU by 35.77 %. The protein with the highest molecular weight of 63.9 kDa (6) found in barley albumins were degraded during the entire malting process by 66.74 %.

Two-dimensional gels of the unmalted (a), germinating (b) and malted (c) albumin fraction are shown in Figure 2B. The degrading protein spots in the range of 10–20 kDa concur with the results obtained with the Protein 80^+ LabChip. In the area of 20–30 kDa two small spots could be detected, which correspond most likely with the area 2 of the LabChip results. There were no increasing protein spots observed in the gels.

3.2.2 Globulin fraction

Figure 3A shows the electropherogram of unmalted, germinating and malted samples of the globulin fraction. Several protein peaks were detected. Whereas the proteins with higher molecular weights of 34.7–40.2 kDa (6) and 57.4 kDa (7) were degraded during the malting process by 44.82 % and 28.89 %, respectively, differences could be observed in the lower molecular weight proteins. Proteins with sizes of 8.8 kDa (1), 25.4–26.9 kDa (4) and 29.2 kDa (5) increased during the first three days of germination by 40.98 %, 21.0 % and 16.67 %, respectively. After kilning levels lower than in the raw barley could be observed. Proteins with molecular weights of 13.7 (2) and 16.8 kDa (3) were markedly degraded by 91.02 % and 79.67 % during the process.

The various types of proteins found with the Lab-on-a-Chip analysis could not be detected on the two-dimensional gels, as shown in Figure 3B. A large degrading protein spot together with a disappearing spot above in the area of 50 kDa could be observed. The protein spot at the bottom of the gel disappeared during the malting process. This protein is most likely the 13.7 or 16.8 kDa-protein, detected with the LabChip.

3.2.3 Prolamin (hordein) fraction

The electropherogram from Lab-on-a-Chip analysis of hordein proteins are shown in Figure 4A. Protein peaks ranging from 13.3–33.0 kDa and from 63.5–92.5 kDa could be observed. Both peak areas were entirely degraded. Already after the germination time of three days no peaks were detected. In Figure 4B the two-dimensional gel images from unmalted, germinating and malted samples can be seen. Some protein spots with molecular weights of 15 kDa and several spots of about 60–70 kDa were detected (Fig. 4B, picture a), which is in agreement with proteins detected with Lab-on-a-Chip analysis. All proteins seem to be entirely degraded during the malting process since the intensity of spots in the germinating sample (Fig. 4B, picture b) was weak and no proteins were visible in the malted sample (Fig. 4B, picture c).

3.2.4 Glutelin fraction

Figure 5A shows the electropherogram from Lab-on-a-Chip analysis of the glutelin fraction. Protein peaks with molecular weights of 35.1 and 64.0 kDa and an area reaching from 42.1 to 46.9 kDa could be observed. Due to the fractionation solution not many glutelins could be extracted, which leads to small peak heights below 10 FU. These observations could be confirmed with the two-dimensional gel electrophoresis (Fig. 5B), were the intensity of proteins is very weak (Fig. 5B, picture a). However, all proteins seem to be entirely degraded after the malting process, as there were no peaks (Fig. 5A, green line) and no spots (Fig. 5B, picture b) detected.

3.4 Free and total amino acid composition of unmalted and malted barley

In Table I the free amino acid composition of unmalted and malted barley are specified. Glutamic acid (0.140 mg/g barley) was found to be the major free amino acid in unmalted barley. In addition, aspartic acid (0.120 mg/g barley), alanine (0.078 mg/g barley) and phenylalanine (0.068 mg/g barley) were also present in high amounts in the unmalted barley kernel. Furthermore, no free methionine could be detected. Proline (3.421 mg/g barley) was existent in the highest concentration of malted barley, followed by phenylalanine (0.723 mg/g barley), arginine (0.722 mg/g barley), leucine (0.555 mg/g barley), valine (0.533 mg/g barley) and threonine (0.530 mg/g barley). Results clearly show an increase in all free amino acids in barley after the malting process. Especially free methionine content increased from 0 to 0.120 mg/g barley during malting. And also proline, threonine, leucine, tyrosine and isoleucine increased significantly by 98.39 %, 96.79 %, 96.76 %, 96.43 % and 95.83 %.

In Table I also total amino acid composition of unmalted and malted barley is shown. Glutamic acid is by far the amino acid with the highest amount (17.490 mg/g barley) in unmalted barley. Proline, followed by leucine and aspartic acid are also present in relatively high amounts of 8.692, 5.425 and 4.917 mg/g barley, respectively. Amino acid composition of malted barley is comparable to unmalted barley, although a slight decrease in total amount of amino acids could be detected. Considering a maximum 5 % error in the method used, following amino acid levels are significantly increasing during malting: tyrosine (by 17.71 %), aspartic acid (by 16.16 %), cysteine (by 11.76 %) and proline (by 11.35 %). Only glutamic acid decreased significantly by 20.36 % during the process.

4 Discussion

4.1 Total protein analysis of barley

A comparison of the free amino acid content in unmalted and malted barley (Table I) revealed an increase in all amino acids during the malting process. This is due to the fact of hydrolysis of the native protein, which gives both high molecular weight protein breakdown products and low molecular weight protein breakdown products (peptides and amino acids) [21]. Quantitatively glutamic acid was the amino acid present in the highest amount in the unmalted sample, which is in agreement with previous studies, where glutamic acid was found to be the major free amino aicd of ungerminated and germinated wheat [22]. In comparison to this the amino acid proline is present in the highest concentration in the malted sample, since its level increased by over 90 % during malting. This is due to the fact that glutamic acid, glutamine and proline play a key roll in amino acid metabolism, since the major proportion of nitrogenous substances originates from these three amino acids. In addition, proline is known to be a good nutrient for the embryo and both proline and glutamic acid are able to enter the embryo faster than other amino acids [23].

Total amino acid composition of unmalted barley (Table I) corresponds qualitatively to that reported by Lásytity [1] and is high in glutaminc acid and proline. A comparison of the unmalted (77.896 mg/g) and malted (77.908 mg/g) total amino acid amounts did not show differences. According to Briggs et al. [24] there is no net loss or gain of nitrogen in barley grain during malting, apart from substances leached during steeping. Glutamic acid was the only decreasing amino acid during malting. This can probably be attributed to the fact that glutamic acid is involved in the metabolism of the production of other amino acids, such as aspartic acid, alanine, isoleucine, tryptophan and threonine, and can be changed into proline in the mitochondria of barley embryos. Proline, along with tyrosine, aspartic acid and cysteine were increasing during malting, which supports the high increase of free proline as described above. An explanation for the high proline increase could be that the different amino acid composition of the protein fractions is relevant, because storage proteins (hordein and glutelin fraction), which were degraded first during malting, are rich in proline. Metabolically active proteins, which belong to the albumin and globulin fraction, do not have high proline levels [1]. During malting mainly storage proteins are degraded, which leads to an increased free amino acid amount, particularly proline.

4.2 Protein frations

4.2.1 Albumin fraction

Albumins and globulins are distinguished as metabolically active proteins in the barley grain [1]. Thus, an increase in these fractions during the malting process was expected. Proteins with the molecular weights between 34.9 to 56 kDa in the albumin fraction were increasing during the malting process, probably due to protein synthesis and the release of some latent proteins and/or the hydrolysis of insoluble hordeins [3]. An 11 kDa protein is detected in area 1 (Fig. 2) and was found to possibly be an isoenzyme of β-amylase [1]. The albumin fraction also contains storage proteins, one with the molecular weight of ~40 kDa, which is commonly mentioned in the literature as protein Z [3, 25]. In Figure 2 A, 40 kDa-protein is shown in area 3, which is decreasing during malting, but still present in a significant amount. This protein is known to act like a storage protein, is resistant to thermal and proteolytic modification and is also present in significant amounts in beer [25]. Another albumin protein,which is known as lipid transfer protein 1 (LTP1) [3, 26], was found to have a mass of 9.983 kDa and is a soluble protein located in the aleurone layer of barley. This protein is known to be surface-acitve and is frequently found in high amount in beer foam. Our results revealed a decreasing 9.4 kDa protein, which is most likely the lipid transfer protein. The decrease is probably due to Maillard-reactions that occur during kilning [26].

4.2.2 Globulin fraction

The globulin fraction consists of metabolically active proteins and storage proteins as well [1, 5]. Our results clearly show increases in the low molecular weight proteins and decreases in the higher molecular weight proteins (Fig. 3), indicating enzymatic as well as storage function of some proteins. The globulin storage proteins are located in the embryo and outer aleurone layer of the endosperm and show similarity to the 7S vicilins of legumes [5]. They are called 7S globulins and have molecular weights of 50–60 kDa [27]. Lab-on-a-Chip analysis revealed a protein peak with the molecular weight of 57.4 kDa, which is decreasing during malting. This is indicative of a storage function of this protein, which is probably degraded to supply the embryo during germination. Similar proteins related to legumins have been reported by Shewry and Halford [5]. They are present in the starchy endosperm of wheat and consist of large (~40 kDa) and small (~22–23 kDa) polypeptide strains. It is likely that these are present in barley as well. Proteins with molecular weights of 34.7–40.0 kDa could be detected (Fig. 3). Since these are decreasing, they may also function as storage globulins. Proteins with molecular weights below 29 kDa are increasing, which is indicative of increased enzymatic activity.

4.2.3 Prolamin (hordein) fraction

Prolamins are the main storage protein fraction of barley and contain low molecular weight proteins. According to their electrophoretic mobility they have been divided into B, C-, D- and γ-hordeins [1]. Skerrit and Janes [17] divided B-hordeins into three subgroups, the B1, B2 and B3 hordeins. The molecular weight value for the B1-hordein of ~31 kDa was reported, based on their amino acid residues [15]. Lab-on-a-Chip analysis revealed a protein with a molecular weight of ~28 kDa, which most likely belongs to the B-hordeins. For the C-hordein fraction values between 55–70 kDa have been reported based on SDS/PAGE analysis [15]. Our results clearly show the largest peaks in the area ranging from 63–92 kDa (Fig. 4A and 4B), where probably not only the C-hordeins fall into, but also D-hordeins (HMW proteins). D-hordeins, which are detected in barley are found to be similar to the ones of wheat glutenin, which have molecular weight values of 83–88 and 67–74 kDa [15], whereas values for barley D-hordeins of 90 kDa [28] and 96 kDa [16] have also been reported. Minor proteins with molecular weights of 15 to 20 kDa (Fig. 5a and 5b) might belong to A-hordeins, which have also been reported by Celus et al. [29] and may be albumins or globulins or breakdown products of larger hordeins rather than true hordeins [30]. Both, Lab-on-a-Chip analysis and two-dimensional gel electrophoresis clearly show a degradation of hordeins during the malting process, which supports the knowledge of cereal prolamins as their function as storage proteins, which supply the embryo during germination with peptides and amino acids [8].

4.2.4 Glutelin fraction

Barley glutelins are known as HMW storage proteins [1]. Depending on the extraction solvent, residues of other fractions remain and can be observed in the glutelin fraction. According to Howard et al. [8] especially B-, C- and D-hordeins remain unextracted, if the previous hordein extraction was carried out under reducing conditions. Our results (Fig. 5A) indicate that C-hordeins with the molecular weight above 63 kDa are present in our glutelin fraction. Proteins with molecular weights of 35 and 42–46 kDa could also be detected, which also have been reported by Weiss et al. [28]. All glutelin proteins appear to be entirely degraded during the malting process (Fig. 5). This is in agreement with Weiss et al. [28], who found fast decreases during the first five days of the malting process.

Table II shows a summary of proteins found in barley including their function. In conclusion this study reveals an understanding of the protein changes taking place during malting of barley. In general a degradation of the proteins to small peptides and amino acids could be observed in the all four fractions with differences in the albumin and globulin fraction, in which some proteins also increased in amount. This is due to the fact that these represent most likely metabolically active proteins. In addition, results show that the Lab-on-a-Chip technique was successfully applied in the characterisation of protein fractions and their behaviour during the malting process.

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6 References

1. Lásztity, R.: Barley proteins: The chemistry of cereal proteins, Second edition, CRC Press, Boca Raton, Florida, 1996, pp. 159-183.

2. Evans, D. E. and Hejgaard, J.: The impact of malt derived proteins on beer foam quality. Part I. The effect of germination and kilning on the level of Protein Z4, Protein Z7 and LTP1, J. Inst. Brew., **105** (1999), pp. 159-169.

3. Silva, F., Nogueira, L. C., Gonçalves, C., Ferreira, A. A., Ferreira, I. M. P. L. V. O. and Teixeira, N.: Electrophoretic and HPLC methods for comparative study of the protein fractions of malts, worts and beers produced from Scarlett and Prestige barley (Hordeum vulgare L.) varieties, Food Chem., **106** (2008), pp. 820-829.

4. Evans, D. E. and Sheehan, M. C.: Don't be fobbed off: The substance of beer foam - a review, J. Am. Soc. Brew. Chem., **60** (2002), pp. 47-57.

5. Shewry, P. R. and Halford, N. G.: Cereal seed storage proteins: structures, properties and role in grain utilization J. exp. Bot., **53** (2002), pp. 947-958.

6. Shewry, P. R., Miflin, B. J. and Kasarda, D. D.: The structural and evolutionary relationships of the prolamin storage proteins of barley, rye and wheat, Phil. Trans. R. Soc. Lond. B, **304** (1984), pp. 297-308.

7. I'Anson, K. J., Morris, V. J., Shewry, P. R. and Tatham, A. S.: Small-angle x-ray-scattering studies of the C hordeins of barley (Hordeum vulgare), Biochem. J., **287** (1992), pp. 183-185.

8. Howard, K. A., Gayler, K. R., Eagles, H. A. and Halloran, G. M.: The relationship between D hordein and malting quality in barley, J. Cereal Sci., **24** (1996), pp. 47-53.

9. Brennan, C. S., Smith, D. B., Harris, N. and Shewry, P. R.: The production and characterisation of Hor 3 Null lines of barley provides new information on the relationship of D hordein to malting performance, J. Cereal Sci., **28** (1998), pp. 291-299.

10. Garcia-Villalba, R., Cortacero-Ramirez, S., Segura-Carretero, A. and Fernandez-Gutierrez, A.: Free-zone capillary electrophoresis analysis of hordein patterns at different stages of barley malting, J. Agric. Food Chem., **54** (2006), pp. 6713-6718.

11. Koehler, S. M. and Ho, T.-H. D.: A major gibberellic acid-induced barley aleurone cysteine proteinase which digests hordein, Plant Physiol., **94** (1990), pp. 251-258.

12. Molina-Cano, J.-L., Polo, J. P., Romagosa, I. and MacGregor, A. W.: Malting behaviour of barleys grown in Canada and Spain as related to hordein and enzyme content, J. Inst. Brew., **110** (2004), pp. 34-42.

13. Shewry, P. R., Hill, J. M., Pratt, H. M., Leggatt, M. M. and Miflin, B. J.: An evaluation of Techniques for the extraction of hordein and glutelin from barley seed and a comparison of the protein composition of Bomi and Risø 1580, J. exp. Bot., **29** (1978), pp. 677-692.

14. Shewry, P. R., Kreis, M., Parmar, S., Lew, E. J.-L. and Kasarda, D. D.: Identification of γ-type hordeins in barley, FEBS Letters, **190** (1985), pp. 61-64.

15. Shewry, P. R. and Tatham, A. S.: The prolamin storage proteins of cereal seeds: structure and evolution, Biochem. J., **267** (1990), pp. 1-12.

16. Celus, I., Brijs, K. and Delcour, J. A.: The effcts of malting and mashing on barley protein extractability, J. Cereal Sci., **44** (2006), pp. 203-211.

17. Skerritt, J. H. and Janes, P. W.: Disulphide-bonded gel protein aggregates in barley: quality related differences in composition and reductive dissociation, J. Cereal Sci., **16** (1992), pp. 219-235.

18. Briggs, D. E., Hough, J. S., Stevens, R. and Young, T. W.: The biochemistry of malting grain: Malting and Brewing Science, Second edition, Chapman & Hall, London, U.K., 1981, pp. 57-107.

19. Brijs, K., Trogh, I., Jones, B. L. and Delcour, J. A.: Proteolytic enzymes in germinating rye grains, Cereal Chem., **79** (2002), pp. 423-428.

20. Moore, S. and Stein, W. H.: Chromatographic determination of amino acids by the use of automatic recording equipment, Methods Enz., **6** (1963), pp. 819-831.

21. Kunze, W.: Raw materials: Technology brewing and malting, 7th edition, Versuchs- und Lehranstalt für Brauerei, Berlin, Germany, 1996, pp. 33.

22. Chittenden, C. G., Laidman, D. L., Ahmad, N. and Wyn Jones, R. G.: Amino acids and quaternary nitrogen compounds in the germinating wheat grain, Phytochem., **17** (1987), pp. 1209-1216.

23. Mandl, B., Wullinger, F., Wagner, D. and Piendl, A.: Effect of germination time on the amino acids of malt, MBAA-Technical Quaterly, **9** (1972), pp. 109-116.

24. Briggs, D. E., Hough, J. S., Stevens, R. and Young, T. W.: The technology of malting and kilning: Malting and Brewing Science, Second edition, Chapman & Hall, London, U.K., 1981, pp. 145-192.

25. Hejgaard, J., Rasmussen, S. K., Brandt, A. and Svendsen, I.: Sequence homology between barley endosperm protein Z and protease inhibitors of the α1-antitrypsin family, FEBS Letters, **180** (1985), pp. 89-94.

26. Perrocheau, L., Bakan, B., Boivin, P. and Marion, D.: Stability of barley and malt lipid transfer protein 1 (LTP 1) toward heating and reducing agents: Relationships with the brewing process, J. Agric. Food Chem., **54** (2006), pp. 3108-3113.

27. Burgess, S. R. and Shewry, P. R.: Identification of homologous globulins from embryos of wheat, barley, rye and oats, J. exp. Bot., **37** (1986), pp. 1863-1871.

28. Weiss, W., Postel, W. and Görg, A.: Qualitative and quantitative changes in barley seed protein patterns during the malting process analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with respect to malting quality, Electrophoresis, **13** (1992), pp. 787-797.

29. Celus, I., Brijs, K. and Delcour, J. A.: The effects of malting and mashing on barley protein extractability, J. Cereal Sci., **44** (2006), pp. 203-211.

30. Baxter, E. D.: Hordein in barley and malt. A review, J. Inst. Brew., **87** (1981), pp. 173-176.

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Appendix

Table I **Free and total amino acid composition of unmalted and malted barley (mg amino acid / g barley grain flour [dry wt])**

	Free amino acids		Total amino acids	
	unmalted	**malted**	**unmalted**	**malted**
Lysine	0.030	0.266	3.170	3.475
Histidin	0.039	0.331	2.524	2.363
Arginine	0.056	0.722	4.513	4.725
Aspartic acid	0.120	0.264	4.917	5.865
Threonine	0.017	0.530	2.636	2.688
Serine	0.024	0.376	3.081	2.981
Glutamaic acid	0.140	0.263	17.490	14.532
Proline	0.055	3.421	8.692	9.805
Glycine	0.032	0.091	3.641	3.630
Alanine	0.078	0.308	3.778	3.834
Cysteine	0.031	0.076	0.375	0.425
Valine	0.045	0.533	4.363	4.614
Methionine	0	0.120	1.804	1.706
Isoleucine	0.013	0.312	2.737	2.804
Leucine	0.018	0.555	5.425	5.296

Table II **Summarizing table of barley proteins found in all four protein fractions**

fraction	size [kDa]	function	probable protein
albumins	9.4	decreasing protein due to Maillard-reactions	LTP1
	11.0	enzyme	isoenzyme of β-amylase
	34.9–56.0	protein synthesis/hydrolysis of insoluble hordeins	
	40.0	storage	protein Z
globulins	29.0	storage	similar to legumin (small polypeptide strain)
	34.7–40.0	storage	similar to legumin (large polypeptide strain)
	57.4	storage	7S globulin
prolamins	15.0–20.0	storage	protein breakdownproducts
	28.0	storage	part of B-hordeins
	63.0–92.0	storage	C-hordeins+D-hordeins
glutelins	35.0	storage	
	42.0–46.0	storage	

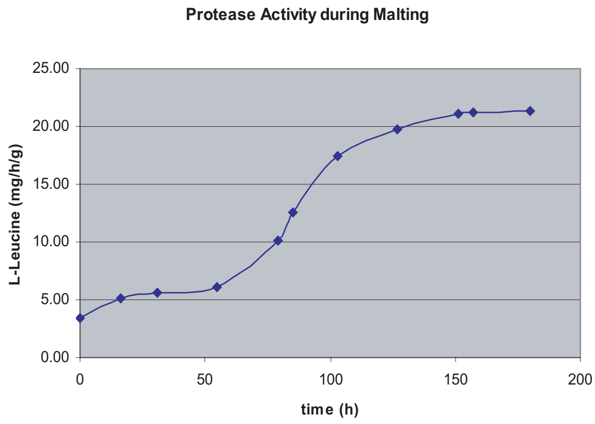

Figure 1 Proteolytic activity (mg L-leucine/h/g) of barley during the malting process in hours. Measurements taken of unmalted (0 h), after first steeping stage (16 h), after steeping (31 h), after first day of germination (55 h), after second day of germination (79 h), during third day of germination (85 h), after third day of germination (103 h), after fourth day of germination (127 h), after fifth day of germination (151 h), after germination (157 h) and after kilning (180 h)

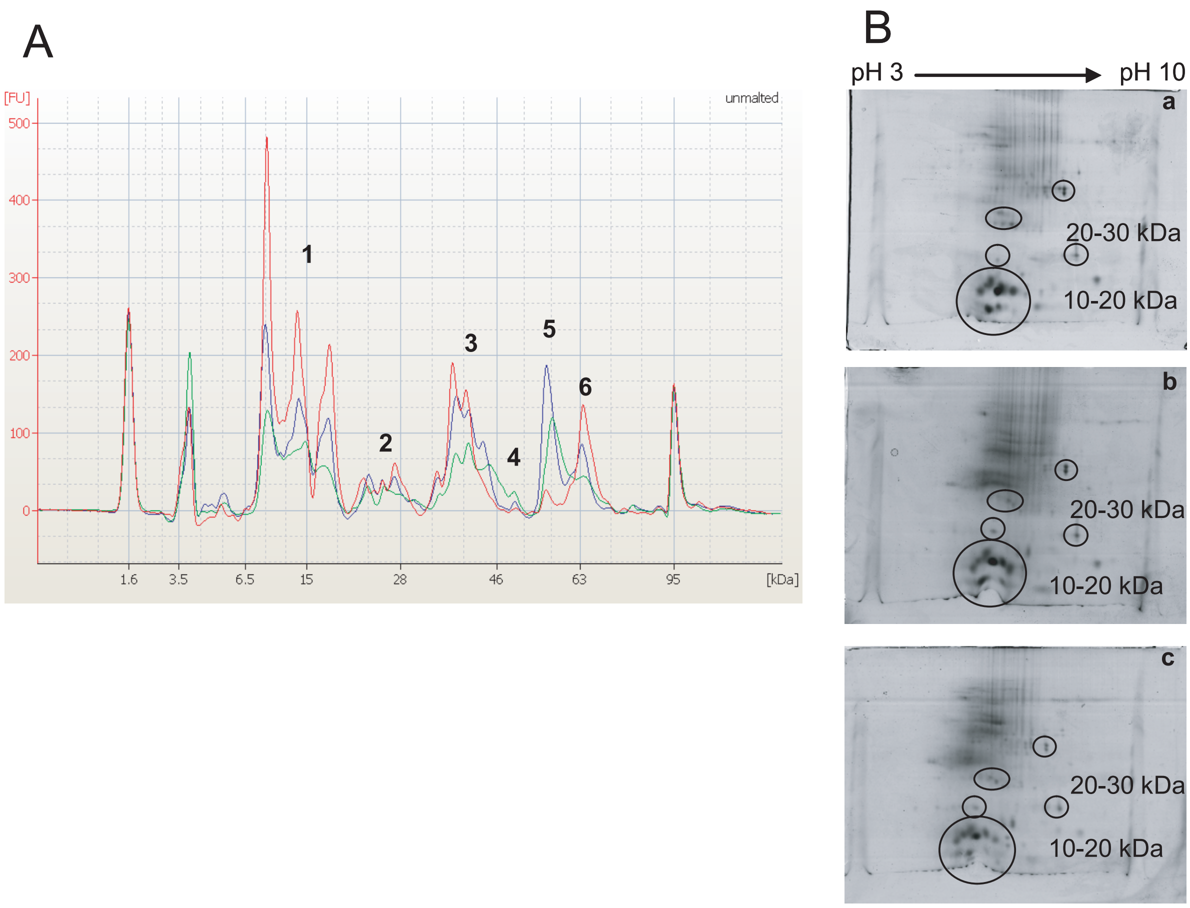

Figure 2 (A) Unmalted (red), germinating (blue) and malted (green) barley albumin (Protein 80+ LabChip). (B) Two-dimensional gel electrophoresis images of unmalted (a), germinating (b) and malted (c) barley albumin

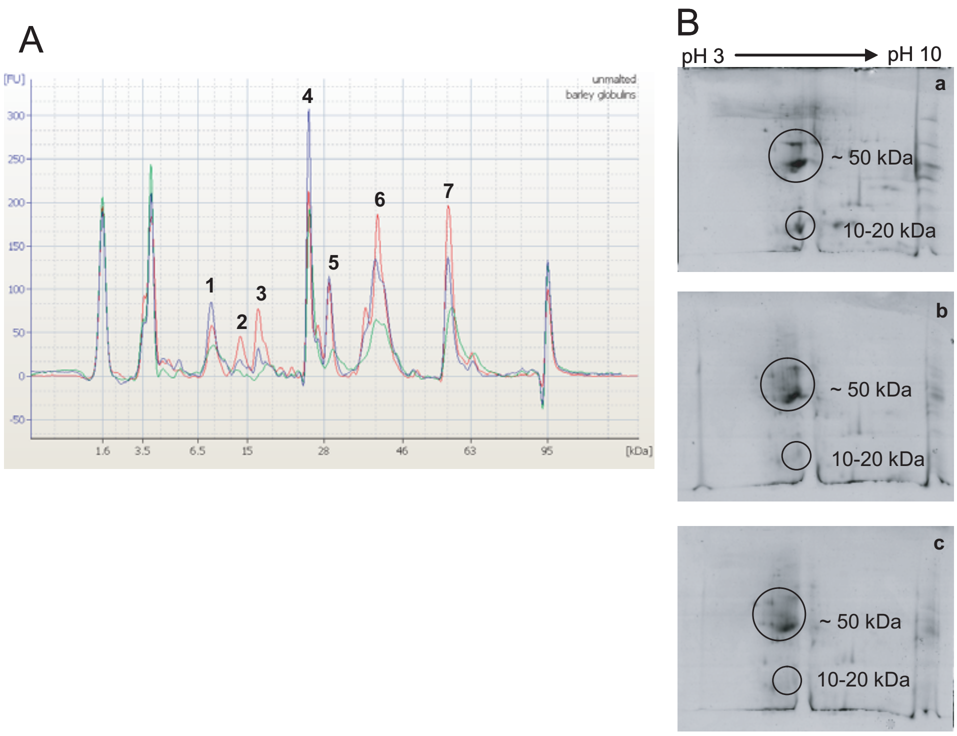

Figure 3 (A) Electropherogram of unmalted (red), germinating (blue) and malted (green) barley globulin fraction (80^{+} Protein LabChip)
(B) Two-dimensional gel electrophoresis images of unmalted (a), germinating (b) and malted (c) barley globulin fraction

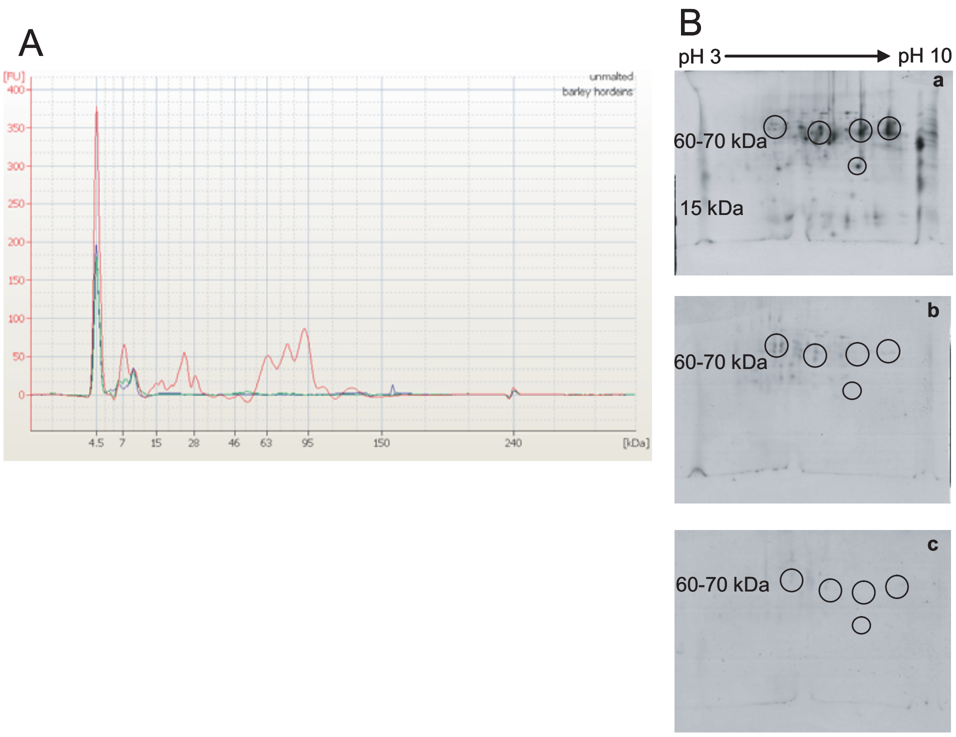

Figure 4 (A) Electropherogram of unmalted (red), germinating (blue) and malted (green) barley prolamin (hordein) fraction (Protein 230^{+} LabChip)
(B) Two-dimensional gel electrophoresis images of unmalted (a), germinating (b) and malted (c) barley prolamin (hordein) fraction

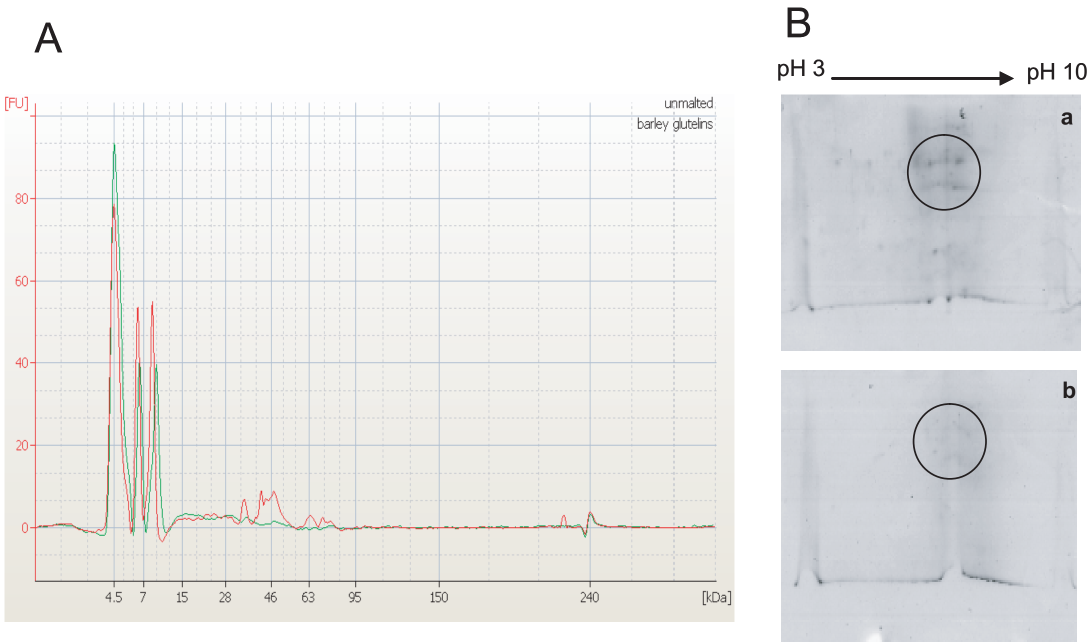

Figure 5 **(A) Electropherogram of unmalted (red) and malted (green) barley glutelin fraction (Protein 230⁺ LabChip)**
(B) Two-dimensional gel electrophoresis images of unmalted (a) and malted (b) barley glutelin fraction