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Proteomics of flour from domesticated varieties and wild barley reveals differences in their hordein compositions

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ABSTRACT

Proteomic studies in barley have been predominantly focused on malting barley for the brewing industry. In this study, protein contents and detailed protein compositions of grains of Wild Barley (WB) (*Hordeum spontaneum*) and two varieties of domesticated barley (*Hordeum vulgare),* Golden Promise (GP), bred for brewing, and PS3, bred for food, were investigated to reveal compositional differences in the hordeins, the major storage proteins of barley. For proteomic profiling a sensitive LC-MS/MS Orbitrap system was used in combination with a one-step denaturing and reducing phenol extraction. The extracts were subjected to label-free bottom-up proteomics. Differences were revealed not only in the overall composition of groups of storage proteins, but also in individual hordeins. WB and PS3 contained higher amounts of D-hordein than GP, while GP had higher levels of B-hordein. Furthermore, the differences in protein composition were evaluated to identify links between the proteome and genotypes' use case and the transition from wild barley.

1. Introduction

Cultivated barley *(Hordeum vulgare)* is well known for its use in beer brewing and feed production. It is currently the 4th most important cereal crop in the world after maize, wheat and rice, and the second most cultivated cereal crop in Europe, after wheat (Giraldo et al., 2019; Pourkheirandish & Komatsuda, 2007). Due to barley's short growing season, it is among the most grown crops in Nordic regions, and as barley also displays robustness to varying growing conditions, it is expected to be quite adaptable to climate change (Daryanto et al., 2016; Jamieson et al., 1995). Importantly, barley has significant environmental and health benefits compared to wheat, because of its lower nitrogen requirement (Poore & Nemecek, 2018) and considerably higher amounts of dietary fibre, respectively (Minaiyan et al., 2014; Schreiber et al., 2020).

It is estimated that 70 % of cultivated barley is used as animal feed globally, while most of the remaining is used for beverages, whereas only approximately 2 % is consumed as food (Baik & Ullrich, 2008). Protein content in domesticated barley grains is around 8–13 % of dry weight, depending on variety and growing conditions (Jaeger et al., 2021; Wang et al., 2010), while in wheat, it varies between 6 and 20 % (Giraldo et al., 2019). Overall barley grain protein content is 8–13 % w/w (Wang et al., 2010).

Barley endosperm contains mainly prolamin (35–45 %) and glutenin (35–43 %) proteins, while the cytoplasmic proteins (mainly albumin and globulin) are enriched in barley bran and germ (Wang et al., 2010). In analogy with the wheat proteins, the prolamins and the glutelins of barley endosperm constitute the gluten proteins, representing the non-water extractable part of the seed proteins, which are held together by a complex network of disulphide bonds within and between prolamins and glutelins, which however have not yet been characterized in barley. In spite of their similarities, the gluten proteins between wheat and barley display profound differences in both structural and functional features, manifesting in a lack of or, at least, a less efficient gluten network in barley compared to wheat, and thereby poorer baking quality of barley flour (Baik & Ullrich, 2008; Lukinac & Jukić, 2022).

In wheat, the glutelins consist of cysteine-rich disulphide-bonded X- and Y-type high-molecular weight glutelin subunits (HMW-GS), as well

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as cysteine-poor low-molecular weight glutelin subunits (LMW-GS). Together they make up the current model for the gluten network in wheat, the main contributor to wheat's good breadmaking quality. The backbone of the model is HMW-GS X and Y-type that form an extending network, despite the lower cysteine content, LMW-GS are still engaged in the disulphide-bond-network with HMW-GS (Wieser et al., 2023). The Osborne prolamin fraction in wheat is called gliadins, which interact with the HMW- and LMW-GS gluten network primarily via hydrophobic interactions. In comparison, barley HMW- and LMW-GS protein equivalents are the B- and D-hordeins, while barley equivalents of wheat gliadins are C- and γ-hordeins {Jaeger et al., 2021 #51. In barley, the major hordein is the B-hordeins (30–45 kDa), constituting 70–80 %, followed by C-hordeins (45–75 kDa) constituting 10–20 %, followed by D- (approximately 105 kDa) and γ-hordeins (35–40 kDa), each consisting <5 % of total hordeins (Jaeger et al., 2021). However, these compositional proportions are largely based on quantifications by SDS-PAGE, and therefore semi-quantitative, and new methods may give new insights into these proportions, also across barley species and varieties, where especially regarding wild barley, a significant knowledge gap remains. Different barley subspecies and varieties have been shown to differ in protein contents, with wild barley having a relatively higher protein content than domesticated barley species and therefore may represent differences in protein compositions and functionality. Wild barley (WB, (*Hordeum vulgare* ssp *spontaneum*) belongs to the grass family *(*Poaceae*)* and is the wild ancestor of domesticated cereal barley (*Hordeum vulgare)*. WB closely resembles the current cultivated 2-row barley, but has slightly narrower leaves, longer stems, longer awns, much smaller grains and cannot be cultivated (Haas et al., 2019). Cultivated barley (*Hordeum vulgare* L. ssp. *vulgare*) is a self-pollinating diploid with 14 chromosomes, present as both 2- and 6-row varieties, and furthermore found as both hulled and hull-less varieties (Schreiber et al., 2020). One of the most popular varieties within brewing and, accordingly, represented in literature, is the Golden Promise (GP) variety. GP is a two-row spring barley, developed in the 1950s by irradiation, with the aim of producing a semi-dwarf variety with good malting characteristics. GP is perceived to have optimal flavor for brewing and is therefore still used, despite newer, higher yielding strains (Schreiber et al., 2020). It is also very agreeable for transformation and easily grows in *in vitro* cultures, which makes it the model plant used by researchers. PS3 is a Danish naked spring barley variety, bred by the Danish breeding company, Agrologica. Naked barley varieties generally present better baking quality than that of hulled varieties, as also observed for PS3. In contrast GP has very poor baking properties (Andrzejczak et al., 2024). Even though Osborne fractionation (Osborne, 1895, 1924) has laid the foundation for categorizing protein fractions in barley and other plants, it will result in overlapping fractions relative to protein profiles, depending on details in the extraction strategy. Apart from details of extraction buffers and inclusion of denaturing or chaotropic agents, the issue of inclusion of reducing agents in the so-called prolamin or glutelin extraction steps are crucial for understanding and comparing results of prolamin and glutelin compositions from different studies, as components of glutelin fraction will be extracted together with the prolamins in case in inclusion of reducing agents in the prolamin extraction step. Accordingly, newer extraction procedures are often used instead, where proteins are extracted in a reducing, denaturing one-step procedure, for example including phenol-phase extractions (Dermendjiev et al., 2021). Therefore, even though Osborne fractionation is universally used in cereal seed protein studies, it is now more accepted with a denotation of "the prolamin super family" comprising both prolamins and glutelins, i.e. the hordeins in barley easily identifiable using immunochemistry (Tanner, Blundell, et al., 2013; Tanner et al., 2019) or a proteomic approach(Lexhaller et al., 2019; Mahalingam, 2017). The albumin and globulin fractions in turn consist of mainly of enzymes, metabolic regulators, plant defensins, inhibitors and minor storage proteins (Bahmani et al., 2021; Gubatz & Shewry, 2010). Based on this, proteins from cereals seeds are often divided into three major groups; 1) storage proteins, which involve storage globulins and prolamin super family (in barley as B, C, D and γ-hordeins); 2) structural and metabolic proteins and enzymes, e.g. α- and β-amylases, peroxidases and lipoxygenases; 3) protective proteins and inhibitors, like serpins, amylase/subtilisin inhibitor, and others (Bahmani et al., 2021).

The aim of the present work was to investigate differences in detailed profiles of proteins in barley flour from the genotypes WB, GP and PS3 by quantitative LC-MS/MS proteomics of proteins, using the one step reducing and denaturing phenol-based extraction procedure of Dermendjiev et al. (2021). Special emphasis will be on hordein composition to provide insight into variations in hordein content and composition relative to the functional phenotypes of the three studied genotypes.

2. Materials and methods

2.1. Preparation of barley flour from WB, GP and PS3

Barley *(Hordeum vulgare* L.*)* flour from two different varieties, PS3 and GP, a malting and a baking barley, as well as from the wild barley species, WB *(Hordeum vulgare* L. *subsp. spontaneum (K. Koch) Thell.)*, that was obtained from NordGen, accession NGB7313, were used in the study. The plants were grown in the greenhouse at an average temperature of 21 °C with 16/8 h light intensity in range of 80–110 μE/m^2/s and fertilised with NPK Makro 13-2-23+Mg + TE (76 g/L) with additional Ca (50 g/L) (Pioner Basis Brun, Azelis, Denmark) at Aarhus University facilities, Department of Agroecology, Research Centre Flakkebjerg, Denmark. Grains were harvested, dried for 48 h at 40 °C, milled using a Fidibus 21 mill (KoMo, Austria) and stored at room temperature until further use. Four individual flour samples of each of the barley genotypes were taken and used as replicates in the following workflow. Protein concentrations in the flour samples were initially determined by Dumas according to (Xiao et al., 2025) in technical triplicates of each flour sample with a conversion factor of 5.83. The varieties were chosen based on their differences in end use properties.

2.2. Proteomic workflow

2.2.1. Protein extraction and proteomic sample preparation from flour of WB, GP, and PS3

For extraction of proteins for proteomics, a one-step phenol/SDS-based reducing extraction of total proteins was used (Dermendjiev et al., 2021). Here, 50 mg of each flour sample (WP, GP and PS3) was extracted with 1 mL of freshly prepared reducing sucrose-SDS extraction buffer (100 mM tris-HCl pH 8.0, 30 % (w/v) saccharose, 2 % (w/v) SDS, 0.5 % (v/v) β-mercaptoethanol, 10 mM EDTA, and including the protease inhibitor Thermo Scientific™ A32953 (Dermendjiev et al., 2021) at room temperature (RT) for 5 min. Then, 250 μL of Roti-phenol was added to each sample, vortexed and incubated for 5 min before centrifugation at 12,000×*g* for 5 min at RT. The supernatants were carefully recovered from each of the extracted flour samples. The phenol extraction was repeated on the remaining sample, and both phenol fractions were pooled. Afterwards, the phenol fractions were counter-extracted once using 1 mL of the reducing SDS-extraction buffer, then centrifuged at 12,000×*g* for 5 min at RT and transferred to a new tube. The proteins were precipitated from the combined phenol supernatant by addition of 2.5 volume of 0.1 M ammonium acetate buffer in methanol to each sample. Obtained protein pellets were washed once with 0.1 M ammonium acetate in methanol, and once with ice-cold 70 % (v/v) methanol, centrifuged after each washing step at 4 °C for 2 min at 18,000×*g*, and dried in a Genevac EZ-2 vacuum concentrator at 30 °C. The one-step extractions were carried out in quadruplicate from each of the four flour samples.

The proteomic sample preparation of the phenol-based flour extracts from WB, GP, and PS3 were performed essentially as described earlier (Shabrangy et al., 2018). The washed protein pellets obtained from the one-step phenol-based protein extractions were redissolved in 360 μl

denaturing and reducing sample buffer containing 10 M urea, 20 mM triethylammonium bicarbonate buffer (TEAB) (T7408 from Sigma Aldrich), 22 mM 1,4-dithioerythritol (DTE) (D8255-25G from Sigma Aldrich). Then 40 μl 200 mM 2-iodoacetamide (IAA, Sigma Aldrich) in 20 mM TEAB was added and left in the dark for 20 min. Protein concentration was measured by Bradford according to the instructions of the manufacturer. The extraction yielded a protein recovery of 10.1 ± 1.5 %, 16.1 ± 1.9 % and 12.9 ± 0.4 % for WB, GP and PS3, respectively. A volume corresponding to 200 μg protein per sample were transferred to new tubes. Lys-C mixture (V5111 from Promega) was added in 1–40 dilution (w/w), and the samples were further diluted 5 x in 20 mM TEAB buffer and incubated for 3h at 37 °C. After digestion, the samples were further 2 x diluted and incubated overnight. A C18 reverse phase desalting column (Thermo Scientific) was used for concentration and desalting of the digested samples. The column was initially washed by pure acetonitrile (ACN) and then with 0.1 % v/v (trifluoroacetic acid) TFA in water. Samples were then acidified by adding TFA to 0.01 % v/v final concentration and loaded onto the column. The column was washed with 0.1 % v/v TFA and eluted with 50 % ACN in 0.1 % v/v TFA. ACN and TFA were evaporated using a Vacuum concentrator (Genevac EZ-2) and the digested samples were stored at −18 °C. The purified peptides were resolubilized in 0.1 % (v/v) formic acid in Milli-Q water prior to LC-MS measurements.

2.2.2. Liquid Chromatography–Mass Spectrometry (LC-MS) measurements

Approximately 1 μg of peptides was separated using a Thermo Scientific Dionex Ultimate 3000 RSLC nano LC system with an Easy-Spray PepMap RSLC C18 analytical column (2 μm, 100 Å, 75 μm × 50 cm), coupled to a Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific). LC separation was performed at 55 °C and 300 nL/min using a 105-min gradient from 5 % to 50 % (v/v) 80 % ACN with 0.1 % formic acid (FA), followed by 5 min at 80 % ACN with 0.1 % FA. Buffer A was 0.1 % FA in Milli-Q water. Peptides were ionized via an Easy-Spray source operated at 1.9 kV in positive mode. MS data were acquired in top-20 data-dependent acquisition (DDA) mode with full MS scans at 70,000 resolution (m/z 200), AGC target of 1e6, scan range m/z 350–1600, and maximum injection time of 120 ms. MS/MS scans were acquired at 17,500 resolution using HCD at a normalized collision energy (NCE) of 27 %. Dynamic exclusion was set to 60 s. Unassigned and +1, +7, +8 and > +8 charged precursors were excluded. The intensity threshold was set to 6.3e3. Isotopes were excluded.

2.2.3. Data analysis

Raw data was processed by PEAKS software version 10.6 (Bioinformatics Solutions Inc.). Parent mass error tolerance was set to 20.0 ppm and fragment mass error tolerance to 0.1 Da. The post translational modifications (PTMs) carbamidomethylation was set to fixed. The following PTMs were set to variable, and included deamidation, oxidation, acetylation, pyro-glu formation from Q or E, and phosphorylation with a maximum of 3 per peptide. Data was filtered at 1 % false discovery rate (FDR) (corresponding to a peptide −10lgP score above 26.6) using the "decoy-fusion" method. *De novo* sequencing identified and kept 15734 peptides after filtration. For identification, Peaks software was used to map *de novo* peptides to a database based on Zeng et al. (2020) as well as 20 additional hordeins reported by Tanner et al. (Tanner et al., 2019; Zeng et al., 2020). The filtration parameters were set to protein −10lgP above 20, and a *de novo* score above 50 %. Using Peaks, a total of 2930 proteins were identified with at least one unique peptide. Further processing was done using Spider, allowing for point mutations between sequence and database and resulted in 2941 identified proteins with at least one unique peptide. The proteins were categorized into functional groups based on (Bahmani et al., 2021; Gubatz & Shewry, 2010; Shewry & Halford, 2002). The functions were determined based on information from UniProt (Consortium, 2024). Quantification for Table 1 is based on label free quantification using only top 3 unique peptides to minimize the effect of ion suppression. This, however, can reduce sensitivity for very small or low-abundance proteins.

3. Results

3.1. Total protein content in flour from WB, GP and PS3

Initially, total protein contents of the barley flour samples as measured by Dumas were determined. The protein contents for WB, GP and PS3 were found to be 21.8 ± 0.6 %, 9.9 ± 0.3 %, and 12.3 ± 0.3 %, respectively, thus confirming higher protein contents in WB, followed by PS3 and then GP.

3.2. Protein composition by proteomics in flour from WB, GP and PS3

To explore and compare detailed protein compositions of each of the three barley genotypes, protein from the flours were extracted by one-step phenol-based extraction at reducing conditions to obtain hordeins and other proteins being part of disulphide networks. The phenol extraction was chosen due to it yielding a broader range of proteins and fewer contaminants (Wang et al., 2008; Wu et al., 2014). By label-free proteomics using an Orbitrap mass analyzer, 2941 proteins were identified in total across WB, GP and PS3, as shown in the Venn diagram (Fig. 1a). These proteins were represented by a total of 15,736 unique peptides (Fig. 1b).

Among the identified proteins, 1964 were present in WB, 2527 in PS3, and 2592 in GP. A total of 1761 proteins (81 %) were shared among all three barley genotypes. Additionally, GP and PS3 shared 500 proteins, whereas WB shared only 72 with GP, and 48 with PS3.

A comprehensive list of the most abundant protein entries were classified into functional groups of metabolic, structural, defence and storage (Table S1). After filtering (above $1*10^7$ of total protein abundance in at least one genotype), 14 entries were from storage proteins, 43 proteins categorized as metabolic and structural proteins, and 17 defence proteins were identified. The storage proteins can be further divided into soluble (mainly globulins) and the non-water-soluble proteins (prolamins). The non-water-soluble proteins consist mostly of hordeins, which, due to their high content of proline and glutamine, are insoluble in water (Bahmani et al., 2021). Among the hordeins, D-hordein was found to be most abundant, with the hordeins identified and quantified to be in the range of D-hordein > B3-hordein (3 entries) > B1-hordein (4 entries) > γ1-hordein (4 entries) > C-hordein (2 entries) > γ3-hordein. Using this further categorization, obtained relative protein compositions based on ion abundances are shown in Fig. 2.

The share of soluble globulins relative to total proteins was slightly higher in the domesticated barley varieties (GP, PS3) constituting approximately 12 % as compared with 9 % in WB as seen under the soluble storage proteins group (dark blue). Larger differences were, however, seen between shares of non-water-soluble storage protein group (light blue), with WB having higher abundance of this type of proteins compared to the domesticated varieties, going from 25 % in WB to 18 % and 15 %, in GP and PS3, respectively. The defence proteins are classified as such, because they are part of the stress response mechanisms, either biotic or abiotic. It includes proteins like alpha-amylase inhibitors or serpins (Bahmani et al., 2021). The ratio of defence proteins (green) was lower in WB (30 %), compared with the domesticated varieties, being 40 % for GP and 34 % for PS3, respectively. Proteins classified as metabolic proteins, are proteins related to the homeostasis of the seed and its ability to germinate. WB and PS3 had the highest shares of metabolic proteins, being 24 % and 23 %, respectively, compared to GP at 20 % (purple). This difference was mainly driven by β-amylase (Table S1 and Fig. 2).

Compositional differences in seed proteins between the species/varieties can show a link between their functional suitability for use in relation to food application. In Fig. 3, protein abundances for both most abundant individual proteins, as well as their functional groupings, as

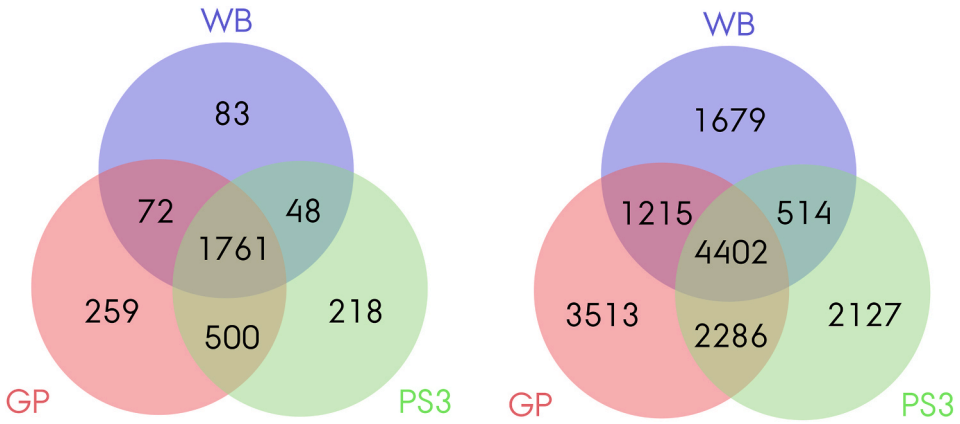


Fig. 1. Venn diagram with sample overlap for protein and peptides, (a) all proteins, (b) peptides. Golden promise (GP) and wild barley (WB).

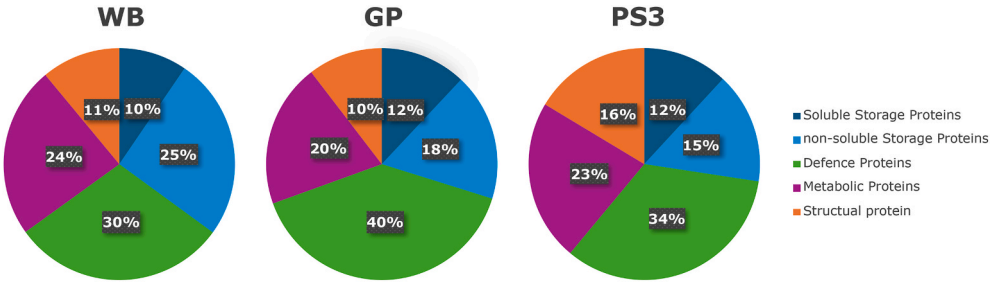


Fig. 2. Relative protein compositions of flour from WB, GP and PS3 categorized into functional groups as based on total ion abundances from proteomics. Storage proteins were divided into soluble (dark blue) and non-soluble fractions (light blue) and the remaining proteins were divided into defence (green), metabolic (purple) and structural (orange).

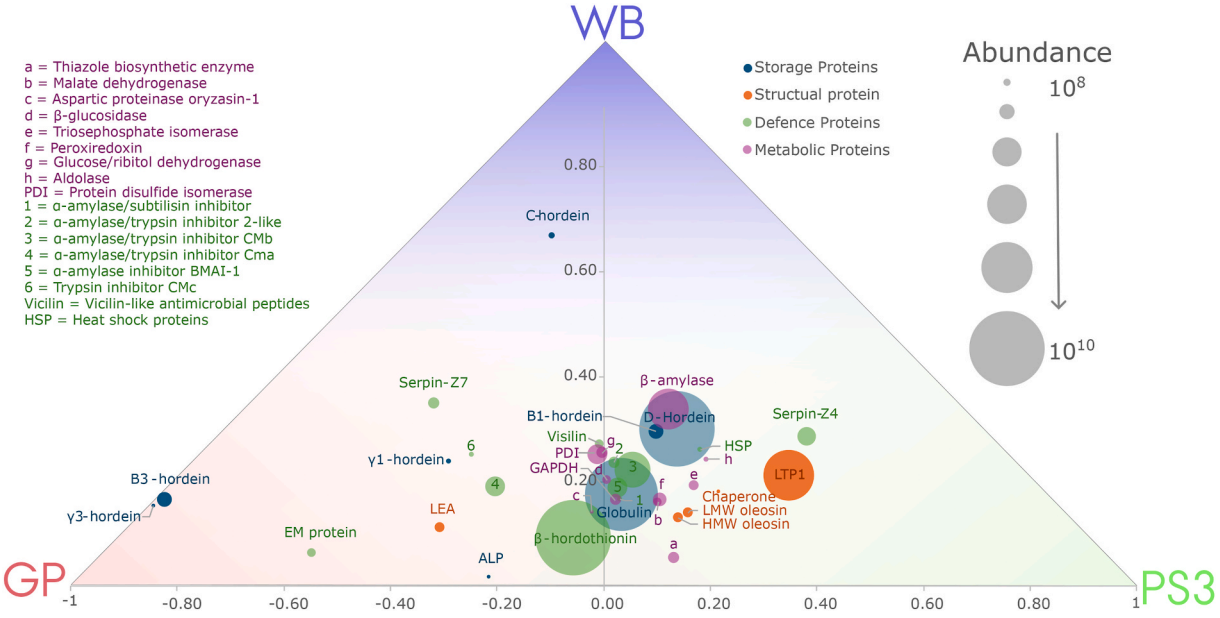


Fig. 3. Modified ternary plot of major individual proteins coloured according to functional group; storage (blue), structural (orange), defence (green), and metabolic proteins (purple). Mean scaled total ion abundance of the proteins is plotted to show the relative expression between the varieties The size of the circle represents total abundance. The further a protein is located toward a corner the more it is expressed in the corresponding variety compared to the others. Equal expression in all three varieties is represented at the coordinate (0, 0.33) in the middle of the triangle. Multiple alleles/isoforms of the same protein are combined for simplicity.

determined for WB, GP and PS3, are illustrated in a ternary plot showing the relative expression between the species/varieties. Among the storage proteins, GP showed an increased relative content of γ-, B3-, and Avenin-like proteins (ALP), as seen by the shift towards the left corner, whereas B1- and D-hordeins were more abundant in WB and PS3, as seen by the corresponding move in the ternary plot. Globulins were almost evenly abundant in PS3 and GP but showed lower abundance in WB. Furthermore, WB contained less γ- and B3-hordeins, as compared to GP.

Serpin contents in barley seeds will vary depending on seed development and stress (Cohen et al., 2019). Here, GP was found to contain higher levels of serpin 4 and lower level of serpin 7, compared to PS3. Lipid binding proteins, like oleosin and Lipid Transfer Protein 1 (LTP1), were found to be more abundant in PS3, compared to WB and GP. Multiple alpha-amylase inhibitors were expressed in high amount, even though alpha-amylase itself was only expressed in a low abundance and therefore also not included in Fig. 3. Beta-amylase on the other hand was highly abundant, especially in WB and PS3.

A closer examination of the storage proteins composition is shown in Fig. 4.

Especially D-hordein was predominant among the storage proteins and constituted almost 50 % of total storage proteins in WB and PS3 (48 % and 45 %, respectively), though less in GP (30 %). GP on the other hand contained 16 % B3-hordein of total storage proteins; a hordein that was almost absent in PS3 and present in 5 % of total in WB. Another major difference was that C-hordein was present to a larger degree in WB (8 %), compared to 2 and 1 % in GP and PS3, respectively. Lastly, it was also observed that WB contained less globulin than the domesticated varieties, at only 27 % compared to 40 % and 42 % in GP and PS3, respectively.

4. Discussion

In this study, protein content and detailed protein compositions of flour from seeds of the three different barley genotypes were investigated. The genotypes included WB (*Hordeum spontaneum*), and two domesticated barley varieties; GP (a malting variety), and PS3 (a variety bred for improved baking qualities). The study aims to reveal correlations between the functional end use properties and the proteome of the three barley types. Barley does not possess the same excellent baking qualities as wheat, partly due to structural differences between the gluten proteins in wheat (prolamins and glutenins), and the hordeins homologs in barley. Wheat gluten proteins are known for cross-linking with each other to form the dough. Barley hordeins' actual contribution in gluten network formation is largely unknown even though the B- and D-hordeins are categorized as glutelins, equivalents of the LMW-GS and HMW-GS in wheat, respectively, and the C- and γ-hordeins are categorized as gliadins (prolamins). In wheat gluten, approximately 50 % of the gluten proteins participate in the disulphide bonded network, while the other 50 % of the gluten proteins exist as non-disulphide-bonded monomeric forms (Li et al., 2020; Wieser et al., 2023). Even though previous studies have hinted at disulphide bonds between D- and B-hordeins (due to the size overlap of B- and γ-hordein on electrophorese gels, more studies should be performed for validation) (Field et al., 1983), it is largely unknown if a similar network exists in barley. Therefore, increased knowledge on especially the different hordeins and their compositional differences in different species and varieties with different functional features is important to advance knowledge on structure-functional relationship at molecular levels in barley flour.

In spite of the notorious poor baking qualities of barley flour compared with wheat, baking varieties of barley do exist. Proteomic studies in barley, however, have been mainly focused on malting barley for the brewing industry. Therefore, this study identifies the difference between domesticated barley developed for brewing and baking as well as wild barley. GP is a widely studied two-row spring barley developed in the 1950s via irradiation to produce a semi-dwarf malting variety with favourable brewing characteristics. In contrast, PS3 is a Danish naked spring barley suited for baking. Unlike PS3, GP has poor baking properties (Andrzejczak et al., 2024). No proteomics studies have so far compared the proteomes to see the differences related to their use. Most proteomics studies in barley have so far been conducted on malting varieties, with the focus on understanding malting properties.

Despite being closely related, both the protein level as well as protein composition was found to vary greatly among the cultivated varieties as well as wild type investigated here. Breeding and domestication of barley to fit different end usage have influenced the grain composition making them distinctly different than their wild relatives. The optimal protein content for malting has been reported to be between 8 and 10 %, whereas the optimal for baking known from wheat is around 12–14 % (Shewry, 1996). The present study revealed that both the PS3 and GP were within the expected protein content for baking and malting, respectively, while a twofold higher protein abundance was observed for WB. The change in protein content is probably due to domestication. Domestication favored traits such as larger grains with higher starch content and greater digestibility, which led to a decrease in protein abundance (Hebelstrup, 2017). Moreover, modern breeding then optimized the domesticated varieties for their end use causing brewing barley (GP) to reach an even lower protein content, as it was shown to greatly influence malting (Hanak et al., 2024). On the other hand, wild species face different selective pressures and aim towards surviving shifting environments, competitions, the need for natural seed dispersal and adaptation to grazers.

In this publication, a deep proteomics analysis of the three genotypes of barley was performed with a focus on change in composition of the proteome relative to hordeins and other storage proteins. It was done using a one-step phenol extraction on flour samples at reducing conditions. A total of 2941 proteins were identified across the three genotypes. The proteins that can be detected vary depending on the method used. In the case of cereals an overlap of 70–80 % has previously been observed when comparing a Tris-HCL and Urea-based extraction. Suggesting that inclusion of multiple extraction methods would be complementary and provide a more comprehensive proteome coverage (Bahmani et al., 2021; Bose et al., 2019; Cilia et al., 2009; Niu et al., 2018). In this study, 1964 proteins were identified in WB, 2527 in PS3, and 2592 in GP, at a FDR of 1 % using a phenol extraction. In other studies of barley a total of 2446 different proteins have been found, using a urea based extraction (Bose et al., 2020), 1185 when using an reducing alkaline water-based extraction (Mahalingam, 2017), or 1497 also using another reducing urea-based extraction protocol (Bahmani et al., 2021). The number of proteins identified here is therefore slightly higher than other deep proteomics studies, which may be due to both the

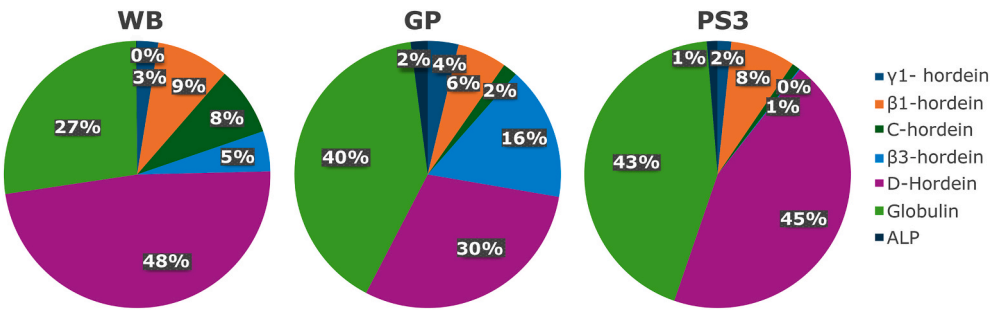


Fig. 4. Pie chart showing relative abundance of storage proteins. γ3-hordein was excluded due to low abundance. Multiple isoforms of the same proteins were combined for simplicity.

differences in extraction conditions as well as in used proteomics equipment.

The distribution of the proteins among the genotypes is shown in the Venn diagram (Fig. 1). Between the genotypes 81 % of proteins were shared, indicating an overall close relationship. It also showed a more similar protein expression between the domesticated varieties compared to WB. Moreover, fewer unique proteins were discovered in WB. However notably, the database used for identification was collected from available protein sequences and could, therefore, be biased towards the extensively researched domesticated varieties leading to a more complete dataset. Moreover, a downing out of low abundance protein might also contribute to less identification in WB, due to the most abundant proteins, such as D-hordein, globulins, β-amylase and β-hordothionin that show very high contribution to the total protein content. A saturation effect in the protein purification could also potentially contribute to the lower number of peptides identified in WB as protein content in the seeds correlate negatively with peptides identified. Comparing the protein composition between the three genotypes, soluble globulins differ between WB, which only contains 9 %, and domesticated at 12 %. A larger difference was seen in the non-water-soluble storage proteins which consist mostly of hordeins, with WB expressing significantly more compared to the domesticated varieties, going from 25 % to 18 % and 15 %, in WB, GP and PS3, respectively. This might indicate that domestication has favored proteins related to a good malting quality as globulin has shown to be beneficial in malting. Moreover, hordeins polypeptides have been shown to correlate negatively with malting quality, with D-hordein having the strongest correlation due to its large amount of polypeptides (Hanak et al., 2024; Howard et al., 1996; Šimić et al., 2007). Contrary, the gluten proteins (hordeins) have the highest correlation with baking quality, impacting properties as viscosity, extensibility and elasticity of dough (Hanak et al., 2024; Li et al., 2020; Shewry et al., 1999).

Seed defence protein composition in WB is lower than in the domesticated varieties at 30 % compared to 40 %, and 34 % in GP and PS3, respectively. However, when compared to the much higher protein content at 21 % in WB seeds a larger amount of defence proteins can still be expected in WB. Interestingly, GP had a lower composition of metabolic proteins at 20 % compared to WB and PS3, mainly driven by containing less β-amylase Table S1 and Figs. 2 and 3 β-amylase together with α-amylase, dextrinase and α-glucosidase constitute the four proteins responsible for catalyzing the hydrolysis of fermentable sugars. Among them β-amylase is the best predictor of diastatic power. Since GP is a malt variety a high β-amylase is expected. PS3 contains the most structural proteins, mainly driven by proteins related to lipids (oleosin and LTP1). WB, GP and PS3 containing 11 %, 10 % and 16 % structural proteins, respectively. LTP1 was shown to be related to foam stability (Steiner et al., 2011).

Hordeins, being the most abundant proteins in barley seeds, contribute to a large variation between the species. Around half the protein in barley seeds depending on nitrogen availability is considered hordeins (Shewry et al., 1999). The composition of hordein has been reported in several studies using different methods such as 1-D gel electrophoresis, ELISA and mass spectrometry (Howard et al., 1996; P.R. Shewry, Kreis, et al., 1985; Steiner et al., 2011; Tanner, Blundell, et al., 2013). Among those, ELISA would be expected to be the most accurate for quantification. However, ELISA has difficulties measuring the exact protein content of hordeins due to antibody epitopes appearing partially or multiple times in hordeins leading to many folds' variation (Tanner, Blundell, et al., 2013). A precise measurement with ELISA furthermore require good controls and so far, especially, D-hordein has proven difficult to purify (Colgrave et al., 2012; Lexhaller et al., 2019; Tanner, Blundell, et al., 2013; Tanner, Colgrave, et al., 2013).

In barley only one D-hordein gene is present contrary to the wheat homolog HMW-GS existing in a x and a y type. Wheat HMW-GS constitute around ≈9 %, in this study D-hordein contributed around 10 %, 5.5 % and 7.5 % in WB, GP and PS3, respectively. This contradicts previous findings where D-hordein content has been estimated by 1-D gel electrophoresis or Elisa to around 1–2 % of hordein content much less that the finding in this study (P. R. Shewry, Kreis, et al., 1985; Wang et al., 2010). Others have estimated D-hordein to be less than 5 % of the hordein content (Galili, 1997; Pont et al., 2020). These findings can, however, be explained partially by the choice of malting barley and extraction method as protein identification and enrichment vary depending on the purification method chosen (Bose et al., 2019) and natural variation between genotypes.

D-hordein dominate the total hordein fraction with 73 %, 56 % and 88 % in WB, GP and PS3, respectively. It was expected that PS3 would contain more D-hordein than GP as it would explain its better baking qualities, however, this fraction represented an unexpectedly large proportion of the total hordein content. Other studies using LC/MS also found D-hordein peptides to be the most abundant when using trypsin as the digestion enzyme in the cultivar Sloop or for both tryptic and chymotryptic in the cultivar Marthe (Lexhaller et al., 2019; Tanner et al., 2019). Total D-hordein in the three barley genotypes matches the range that would be expected for HMW-GS in wheat varieties. Therefore, we can only conclude that it is the other hordeins that are underrepresented in the quantification or that other studies previously have underestimated the D-hordein content. This can partly be explained by hordeins repetitive sequences not containing any tryptic cleaving sites which can partly be mitigated by using top three unique peptides for quantification instead of all peptides. Using only the top peptides will, however, push the qualification towards resembling a molar ratio as only a small part of each protein is represented independent of protein size. B-hordein content in barley optimized for beer production has been measured up to 70 % of the total hordein fraction (Colgrave et al., 2012; Tanner, Colgrave, et al., 2013). The measurement, however, was done by ELISA. Therefore, 70 % B-hordein could be an overestimation. In this study 38 % of the hordeins were B-hordein in GP. WB and PS3 only contain 19 and 14 % B-hordein.

C-hordein (three different C-hordein were pooled) constitute 3 %, 2 % and 11 % of total hordein in GP, PS3 and WT, respectively. Interestingly C-hordeins contain no cysteines and are generally sulfur poor. C-hordein was more abundant in Wild barley compared to the domesticated types. C-hordein can vary in barley from below 10 % of hordeins to 80 % when grown in sulfur deficient environments (Colgrave et al., 2012; Shewry et al., 1999). γ-hordeins (γ1 and γ3 types were combined) constitute 3 %, 4 % and 5 % of total hordeins in WT, PS3 and GP, respectively.

In conclusion, hordein content varies greatly depending on the genotype, extraction method and quantification method. The composition of especially hordein would impact the function of barley as the food ingredient as hordeins are homologs to wheat gluten proteins. Highlighting the importance of breeding in the development of barley with desired functionalities and that these functionalities are reflected in the proteome. However, even PS3, that was breed for baking, does not possess the same desirable properties as wheat, likely due to a structural difference between the wheat gluten proteins and barley's hordeins. Hordeins do exhibit distinct structural and functional features compared to gluten proteins in wheat, which could be due to a different disulphide-bond formation of D- and B-hordeins as compared to HMW- and LMW-GS, and/or altered hydrophobic prolamin interactions via its C- and γ-hordeins compared with the gliadins in wheat. However, this should be further investigated.

CRediT authorship contribution statement

Esben Olesen: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Julia Füreder:** Writing – review & editing. **Leila Afjehi-Sadat:** Writing – review & editing, Methodology, Formal analysis. **Olga A. Oxholm:** Writing – review & editing, Supervision, Funding acquisition. **Ulla Kidmose:** Writing – review & editing,

Supervision, Funding acquisition. **Kim Hebelstrup:** Writing – review & editing, Supervision, Funding acquisition. **Verena Ibl:** Writing – review & editing, Funding acquisition, Conceptualization. **Nina A. Poulsen:** Writing – review & editing, Supervision, Funding acquisition. **Lotte B. Larsen:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2025.107892.

Data availability

Data will be made available on request.

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