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Short Communication

LC-MS/MS Reveals Hordeins Are Enriched in Brewers' Spent Grain

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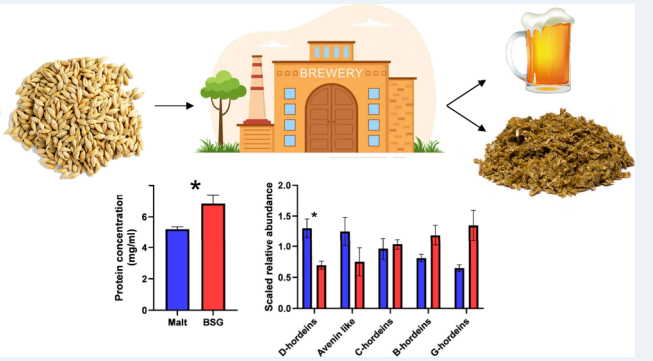
ABSTRACT: Barley is commonly used in malting and brewing, and spent grain is repurposed for other foods. Barley contains gluten proteins called hordeins that cause intestinal damage and disease symptoms if eaten by people with celiac disease and related conditions. While the mashing process in brewing can partially hydrolyze immunogenic epitopes in hordeins, the immunogenic epitope load between the starting malt and spent grain has not been investigated. Herein, we quantified hordeins in commercially available spent grain and from matching malt. Liquid chromatography−mass spectrometry (LC-MS) and sandwich and competitive R5 ELISAs were used for quantification, revealing a higher abundance of gluten proteins in the spent grain product compared with the input malt. Certain hordein subtypes were enriched while others were depleted, and overall protein content was higher in spent grain. This suggests that the mashing process selectively extracts nonprotein components, leaving protein and hordein content elevated in spent grain. The spent grain products tested were not safe for consumers with celiac disease.



Gluten proteins occur in wheat, barley, rye, and related cereal species as members of the prolamin superfamily. These proteins contain immunogenic epitopes that elicit symptoms in celiac disease (CD) and related immune disorders. Eating as little as 50 mg of gluten can cause intestinal damage,[1] which makes food gluten content labels an important source of information guiding food choice for this susceptible subset of consumers.

In barley, C-hordeins resemble wheat omega-gliadins and contain epitopes that are immunogenic for CD. B- and γ-hordeins have fewer immunogenic epitopes than C-hordeins, while D-hordeins are the least immunogenic for CD. While the barley grain protein abundance and proportions depend on genotype and environment, 30−50% of the barley grain proteome are hordeins,[2] the bulk of which are B- (70−80%), followed by C- (10−20%), D-, and γ-hordeins (∼1−2%).[3] These ratios change over the course of malting,[4] and mashing further alters the proteome by removing proteins soluble in hot water and aggregating and precipitating others. The extent to which it affects the net gluten epitope content is unclear.[5]

Food allergen and gluten content labeling regulations differ between countries. The Codex Alimentarius (CDX 118-1979) represents a consensus of food safety practices from different jurisdictions[6] and recommends that gluten quantification be performed using a sandwich enzyme-linked immunosorbent assay (ELISA) using the R5 antibody. This differentiates two categories for the labeling of gluten content: "gluten-free" for foods containing <20 mg/kg gluten or "very low gluten" or similar for food products containing 20−100 mg/kg gluten.[7] The 20 mg/kg threshold has been adopted by many countries, but there remains contention regarding the measurement of gluten content in fermented or hydrolyzed foods, where epitopes may be present but difficult to detect and quantify. After enzymatic or heat-related hydrolysis, protein fragments may not be detectable using a sandwich ELISA as they may not possess the two antigenic epitopes required for detection in the sandwich ELISA.[8] A competitive ELISA is more appropriate in these circumstances as they can detect peptides that contain a single R5 epitope, though recommended assays have known shortcomings when quantifying heated or hydrolyzed proteins.[9]

In the brewing industry, malted barley is exposed to a series of hot water incubations to digest and extract fermentable sugars. This is called the "mash", and after removing the liquid "wort" for beer production the remaining brewers' spent grain (BSG) is food-safe but typically used for animal feed or industrial applications. BSG has recently entered supermarket shelves in the form of brewers' flour and is rich in protein, dietary fiber, B vitamins, minerals, and in some instances, has been advertised as low-gluten and may carry a low- or no-

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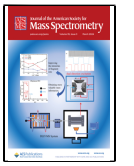

gluten label. Regulations in some jurisdictions state that gluten content measurements can be made using sandwich ELISA, which may not be suitable for BSG due to the high temperatures and enzymatic proteolysis that occur during mashing.

The desirable aspects of BSG have been reviewed recently[2] but do not include reference to immunogenic epitopes. Given the utility of BSG in novel food applications, there is a need to investigate immunogenic epitopes and consider food safety risks. Accordingly, this study investigated the gluten content of malted barley before and after mashing using both competitive ELISA and targeted LC-MS/MS.

METHODS

Samples. A sample of BSG flour was obtained from a commercial manufacturer for this project. To make this BSG flour, BSG was collected, dried, milled, and commercially packaged. A sample of the same malt (Schooner Pale malt, a Munich-type malt) that was used in the production of this batch of BSG flour was also obtained. The malt sample was received, milled using a laboratory ball mill (MM400, Retsch, Germany), and sifted through a 300 μm screen to ensure a fine particle size.

Sandwich and Competitive ELISAs. The RIDASCREEN Gliadin sandwich and, separately, competitive ELISAs were used. Samples were extracted and analyzed in duplicate as per the manufacturer's instructions.[10] The BSG and malt samples were tested alongside a wheat flour sample used as a positive control and seven other rice-based food products as negative controls.

Proteomics Sample Preparation and Analysis. Proteins were extracted from the malt flour and BSG flour by an established method and protocol,[11] using 8 M urea and 0.1 M tris-HCl, pH 8.4, as the extraction solvent. Protein concentration was estimated by the Bradford assay. Both tryptic and chymotryptic digestions were performed on independent extracts.

Discovery proteomics was performed on malt samples to detect and identify hordein proteins using LC-MS instrumentation, conditions, and settings as described previously.[11] Data were searched against a FASTA file consisting of the hordeum genus of proteins from UniProt-KB (accessed 08/2022 and supplemented with additional translated gene models from the IWGSC RefSeq v1 Assembly,[12] as well as those listed on the common Repository of Adventitious Proteins (thegpm.org/crap)). The FASTA file contained 57,925 protein sequences.

Hordein proteins identified at 1% FDR in malt were quantified in scheduled multiple reaction monitoring (MRM) analyses, which were run on tryptic and chymotryptic digests of both malt and BSG.

RESULTS

Gliadin Quantification Using ELISA. The standard extraction process for RIDASCREEN sandwich and competitive trace gluten ELISAs involves a 500x dilution factor. To estimate gluten content in raw barley products, the malt and BSG extracts required an extra 200-fold (100,000x in total) to meet the expected quantitative range (10−270 mg/kg for competitive ELISA, 5−80 mg/kg for sandwich ELISA). BSG had a higher gluten protein content than malt in both cases, though values were only within the quantifiable range for competitive ELISA (Table 1).

Table 1. Gluten Protein Quantification by Competitive and Sandwich ELISAs

sample identity	dilution factor	competitive ELISA gluten measurement (mg/kg)	sandwich ELISA gluten measurement (mg/kg)
malt	100,000	4,598	>16,000
BSG	100,000	32,582[a]	>16,000
wheat flour (+ control)	100,000	41,916	>16,000
rice food (− control)	500	<10	<5

[a]BSG yielded a competitive ELISA result 7 times higher (32,582 mg/kg) than malt, consistent with the idea that mashing selectively removes nonprotein material, leaving BSG with a higher proportion of proteins by mass, some of which contain the R5 epitopes (QQPFP, QQQFP, QLPFP, and LQPFP[13]).

Protein Concentration. Protein content was estimated by the Bradford assay (Figure 1) as 6.8 mg/mL for BSG and 5.2 mg/mL for malt.

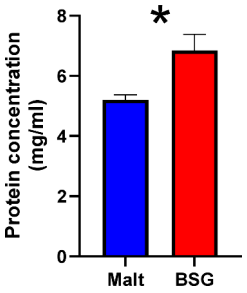

Figure 1. Protein estimation in malt and BSG extracts (*$p < 0.05$, determined using unpaired t test, one-tailed, assuming unequal variance).

Peptide Quantification Overview. Due to the glutamine-rich and repetitive nature of hordein proteins, many hordein-specific peptides are shared between multiple proteins and protein isoforms and repeated multiple times within the same protein sequence. Altogether, 28 tryptic and 35 chymotryptic peptides were monitored, five of which had variable modifications (three deamidation and two oxidized methionine). These appear in a total of 93 barley proteins. Of the 63 targeted peptides, 22 were unique to a single protein, and the remaining 41 peptides identified a further 14 proteins, being present a total of 629 times within the total of 93 proteins.

Quantitative Analysis of Hordein Subtypes. To explore the protein-level changes that are selectively depleted or concentrated in the grain by mashing and lautering, quantified peptides were mapped to the barley proteome in UniProt and categorized into the hordein subtypes: B-hordein, C-hordein, D-hordein, γ-hordein, and avenin-like proteins (ALPs). Relative peptide abundance was determined by creating individual gluten peptide ratios, wherein each peptide MRM peak area was compared to the average peptide MRM peak area (comprising malt and BSG). The peptide ratios were grouped according to gluten subtype and plotted (Figure 2A) as the average value (± SD). This grouping was necessary, as individual gluten peptides often map to multiple protein isoforms within a gluten subtype.

This revealed that BSG comprised a higher proportion of γ-, B-, and C-hordeins and was depleted in ALPs and D-hordeins. Grouping all peptides together shows the average hordein

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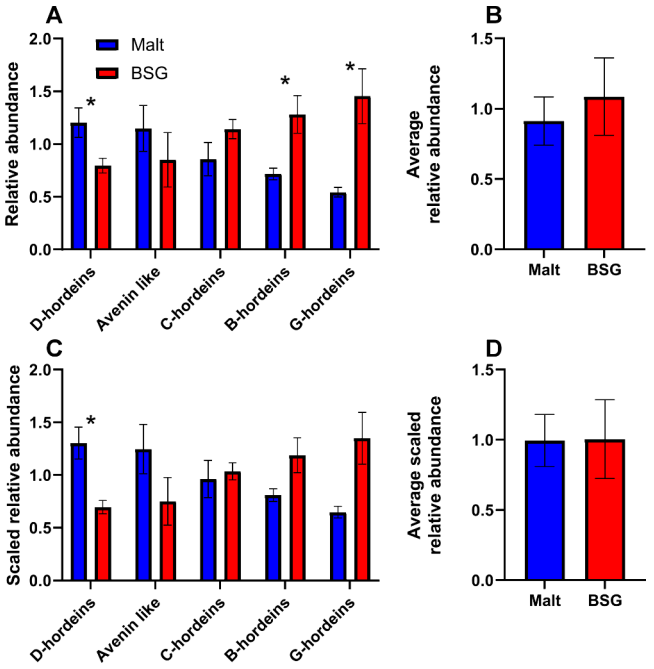

Figure 2. Quantitation of hordein peptides in malt and BSG. (A) Relative abundance of hordein subtypes per gram of starting material. (B) Net hordein content. (C) Relative abundance of hordein subtypes corrected for total protein content. (D) Net hordein content corrected for total protein content (*$p < 0.05$ by unpaired t test).

peptide abundance was approximately 25% higher in BSG (Figure 2B). The γ-hordeins (G-hordeins) were significantly enriched in spent grain (on a grain weight basis).

The proportionally higher hordein content in BSG reflects the higher total protein content. Normalizing malt and BSG peptide abundance values according to protein content (Figures 2C and 2D, scaled by a factor of 6.8/5.2 according to Figure 1) corrects for the difference in total protein content caused by mashing and lautering. Grouping proteins together after correcting for protein content differences revealed no significant difference in the peptide abundance (Figure 2D).

DISCUSSION

This investigation quantified gluten protein content in malt and BSG to reveal how mashing and lautering impact the hordein content. We show how B- and γ-hordeins are proportionally higher in BSG while D-hordeins are removed in the wort, with the net effect being an overall increase in the hordein concentration. When normalized for protein content, the relative hordein content was not significantly different between malt and BSG, though significant protein class-specific trends remained. The higher total hordein content of BSG is largely due to the elevated total protein content and is consistent with the hypothesis that mashing removes water-soluble malt components (starch, sugars, β-glucans, polyphenols, and soluble fiber), while protein accumulates in BSG.

The differences in results among the three measurement techniques demonstrate the complexities and limitations of gluten analytical methods. The sandwich ELISA has a higher sensitivity to low levels of gluten but with a smaller quantifiable range, while 200,000-fold diluted extracts fell within the quantifiable range of the competitive ELISA (Table 1). LC-MRM-MS allowed the relative quantification of dozens of tryptic peptides in malt and BSG, but targeted LC-MRM-MS will not detect peptides that are modified during mashing with glycation as an example of an observed modification. Moreover, extensive hydrolysis will impact the ability of both ELISA and LC-MS to detect gluten or other foodborne proteins. All three techniques rely upon effective extraction before measurement, which is a known challenge for intact proteins in ELISA assays,[9] and protein aggregation may hide/mask antigenic sites (ELISA) or trypsin digestion sites (LC-MS).

The high residual gluten content in BSG shows that it is unsafe for consumers with CD. While sandwich-style ELISAs are sensitive and reproducible and are recommended by the Codex Alimentarius, the competitive ELISA is more appropriate when proteins may contain a single instance of the epitope after cooking and proteolysis as expected during mashing. Both ELISAs showed abundant signal and higher gluten readings for BSG than malt, consistent with LC-MS quantification (Figure 2B).

Drawing broader conclusions about gluten protein content in BSG would benefit from a wider survey of barley genotypes and brewing approaches. This observational study of a single barley genotype and one brewing style revealed no significant hordein reduction. Efforts to lower BSG hordein content may benefit from using adjuncts and incorporating an extended protease rest during brewing. However, a CD-safe BSG would require gluten-free ingredients, such as pseudocereals, to be used instead of traditional barley.

CONCLUSION

LC-MS revealed significant and selective changes to the malt proteome over the course of mashing and lautering, falling unevenly on different hordein subtypes. The partial digestion and removal of proteins and peptides significantly depletes D-hordeins, ALPs, and nonprotein components, thus seemingly enriching γ- and B-hordeins in BSG. BSG is 31% higher in protein content on a dry weight basis compared to the input malt and has a higher net hordein content that approaches significance when measured by targeted LC-MRM-MS. When measured by sandwich and competitive ELISAs, the gluten content was several orders of magnitude above the safety threshold for gluten-free foods.

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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