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What is in a Beer? Proteomic Characterization and Relative Quantification of Hordein (Gluten) in Beer

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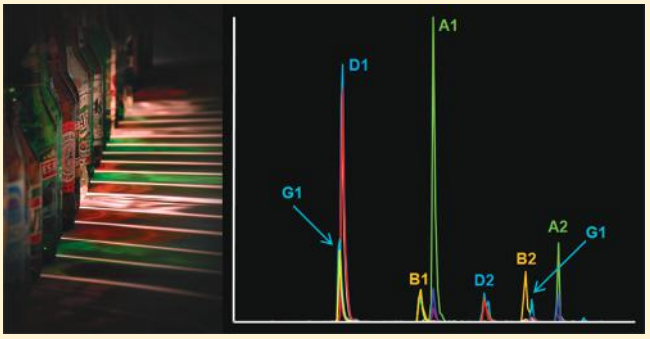
Supporting Information

ABSTRACT: The suite of prolamin proteins present in barley flour was characterized in this study, in which we provide spectral evidence for 3 previously characterized prolamins, 8 prolamins with only transcript evidence, and 19 genome-derived predicted prolamins. An additional 9 prolamins were identified by searching the complete spectral set against an unannotated translated EST database. Analyses of wort, the liquid extracted from the mashing process during beer production, and beer were undertaken and a similar suite of prolamins were identified. We have demonstrated by using tandem mass spectrometry that hordeins are indeed present in beer despite speculation to the contrary. Multiple reaction monitoring (MRM) mass spectrometry was used for the rapid analyses of hordein in barley (*Hordeum vulgare* L.) beer. A selection of international beers were analyzed and compared to the results obtained with hordein deletion beers. The hordein deletion beers were brewed from grains carrying mutations that prevented the accumulation of either B-hordeins (Risø 56) or C-hordeins (Risø 1508). No intact C-hordeins were detected in beer, although fragments of C-hordeins were present in wort. Multiple reaction monitoring analysis of non-barley based gluten (hordein)-free beers targeting the major hordein protein families was performed and confirmed the absence of hordein in several gluten-free commercial beers.



KEYWORDS: hordein, gluten, multiple-reaction monitoring, mass spectrometry, proteomics, quantification

INTRODUCTION

Coeliac disease (CD) is a T-cell mediated enteropathy. In the case of the most studied prolamin, α-gliadin, toxicity is largely mediated by a single glutamine in a single peptide[1,2] that produces a destructive cascade of reactions that eventually damage the small intestinal villi, reducing nutrient absorption and impacting health. Coeliac toxic epitopes for all known prolamin proteins from wheat, barley and rye have now been extensively mapped using unbiased T-cell populations isolated from the peripheral blood of HLA-DQ2$^+$ coeliacs following short-term dietary challenge with wheat, barley and rye.[3] Surprisingly, only three highly immunogenic peptides, derived from α-gliadin (ELQPFPQPELPYPQPQ), ω-gliadin/C-hordein (EQPFPQPEQPFPWQP), and B-hordein (EPEQPIPEQPQPYPQQ), could account for 90% of the coeliac-specific response, elicited by the full complement of wheat, barley and rye proteins. Clinical symptoms of CD include fatigue, diarrhea, abdominal distension, weight loss, anemia and neurological disorders.[4] CD has been associated with increased rates of intestinal malignancy, such as 10-fold increased risk of intestinal cancer, a 3- to 6-fold increase in the risk of non-Hodgkin lymphoma and a 28-fold increased risk of intestinal T-cell lymphoma[5] as well as increased rates of anemia, osteoporosis, neurologic deficits, and additional autoimmune disorders such as diabetes.[6]

The only current treatment for CD is lifelong avoidance of dietary gluten, which consists of a family of similar proteins found in wheat (gliadins, glutenins), rye (secalins), barley (hordeins), and oats (avenins). However, such diets are costly[7] and associated with low fiber and high sugar intakes,[8−10] which in themselves are health risks. Avoidance of dietary gluten leads to a normalization of health statistics in most, but not all coeliacs.[11,12] Approximately 1% of most populations worldwide suffer from coeliac disease, however, up to 50% of adults remain undiagnosed or do not display overt symptoms, termed "the coeliac iceberg".[13,14]

The major families of seed proteins are the albumins, globulins and the gluten-like proteins, collectively called prolamins.[15] The albumins and globulins are widely distributed among flowering plants, but the prolamins are restricted to the grasses.[16] Figure 1 illustrates selected examples of plants belonging to the grass

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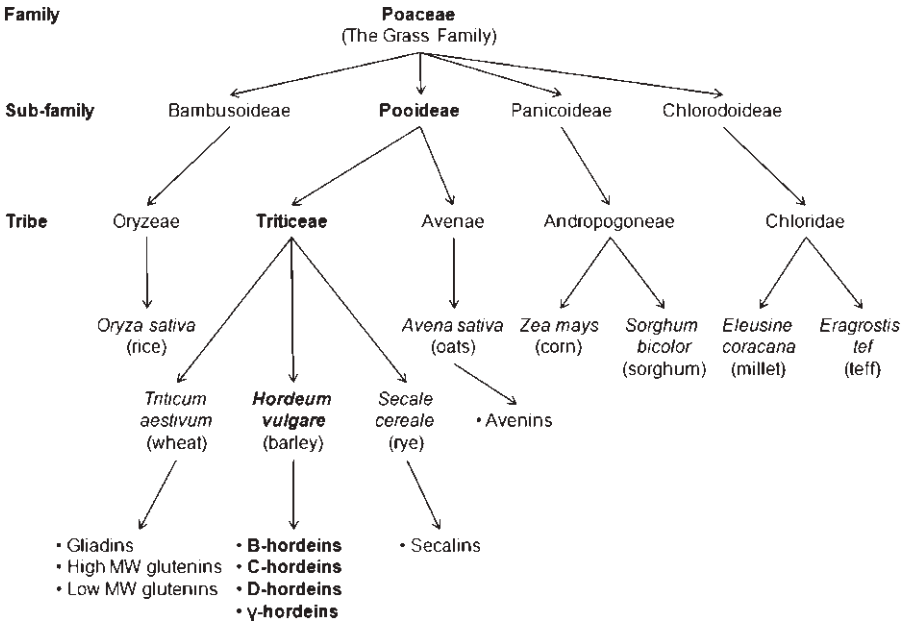

Figure 1. Classification of gluten proteins. Coeliac patients react to toxic peptides produced by members of the Triticae tribe, including *Triticum aestivum* (wheat), *Secale cereale* (rye), and *Hordeum vulgare* (barley). A small percentage of coeliacs exhibit a genuine reaction to avenins from *Avena sativa* (oats). Beer is produced by the brewing and fermentation of sugars derived from cereal grains, most commonly barley and wheat, but rice, corn and oats are often used as adjuncts. Gluten-free beers may also be brewed using sorghum, millet and teff.

family that are used to produce beer. The prolamin proteins produced in each of the Triticeae and Avenae plants are listed. Prolamins, so named because they contain a high proportion of proline and glutamine, resist proteolysis during digestion.[17] The α-gliadin gene was the first prolamin gene to be cloned and much is now known about the role of this protein in coeliac disease.[18] The toxicity of this prolamin is focused on a single peptide 57QLQPFPQPQLPYPQPQS73 with the glutamine residue Q65 being the key amino acid. Mutation of this single glutamine to, for example, a lysine, abolishes the coeliac toxicity of this prolamin.[1] Partially hydrolyzed peptides cross the epithelium and access the lamina propria by an unknown mechanism; here Q65 is deamidated by tissue transglutaminase (tTG) to E65 (glutamate), increasing the immuno-stimulatory potential of the peptide.[19] The negative charge facilitates binding of the peptide to DQ2 (or less commonly DQ8) receptors on the surface of Antigen Presenting Cells, allowing presentation of the peptides to particular gluten-specific, DQ2-restricted, CD4+ T-cells, which are targeted to the intestine. Thus activated, the CD4-T cells undergo clonal expansion and in turn assist the expansion of gluten-specific- and TG2-specific-B-cells with the resultant production of anti-TG2 and antigluten antibodies characteristic of coeliac disease. A cell-mediated Th1 response also occurs, through the secretion of inflammatory cytokines.[20] Thus, a simple protein interaction, facilitated by the introduction of a single negatively charged residue, initiates a series of specific and targeted cascades, ultimately leading to destruction of the intestinal villi in susceptible individuals.

Barley (*Hordeum vulgare* L.) is a widely grown cereal used to produce malt for the brewing and food industry. Malted barley is the main ingredient in beer, supplying the carbohydrate source for fermentation. Unfortunately for coeliacs, barley beer also contains a low, but coeliac-toxic level of hordein (gluten).[21] There has been some speculation about the presence of and/or amount of gluten present in beers. A recent report examining the level of gluten in commercial beers found that the gluten content of 50% of the beers tested, as measured by ELISA, contained less than the guidelines established by Codex Alimentarius Standard (20 ppm gluten).[22] Likewise, in a study of two Italian barley malt beers, only trace levels of hordeins were detected by ELISA, although peptide degradation products of hordeins were detected using LC−MS/MS.[23,24] The hordeins account for half of the barley grain protein,[25] and are composed of four families: the B-hordeins (30−45 kDa; 70% of hordein content) and the C-hordeins (45−75 kDa; 20% of hordein content) dominate the grain hordein, while the D- (105 kDa) and γ-hordeins (35−40 kDa) are minor components.[25] The use of malted sorghum, millet, or buckwheat as gluten-free alternatives for beer production is now common;[26] however, it is difficult to reproduce the quality and the economics of barley beer with these grains.

The WHO standard definition for gluten-free foods adopted by the Codex Alimentarius[27] in 2008 requires that food prepared from cereals such as wheat and barley must contain less than 20 mg/kg (ppm) gluten to be labeled "gluten-free". Most international jurisdictions are adopting similar recommendations; however, in Australia, FSANZ[28] adds the caveat that barley malt cannot be used to prepare gluten-free food. A wide range of analytical methods for gluten measurement have been assessed over the past few decades.[29−31] Only two enzyme-linked immunosorbent assay (ELISA) protocols have been internationally ring-tested: (1) the Mendez R5 mAb, raised against rye prolamins, which recognizes the epitopes QQPFP, QQQFP, LQPFP, QLPFP[32] and (2) the Skerritt mAb raised against ω-gliadins, which detect the epitopes PQPQPFPQE and PQQPPFPEE.[33] Both antibodies have problems, in that they under- and over-represent individual hordein families (Tanner, Pers. commun.). The R5 antibody is unable to accurately detect and quantify barley gluten (hordeins) in beer.[34] The sandwich R5 ELISA is able to quantify native and heated gluten, but overestimates hordeins.[35,36] The use of competitive ELISA is more sensitive, but it is difficult to match the prolamin being measured with the competitor prolamin

supplied in the ELISA kit. Thus, there is an overarching need for accurate quantitative methods to assess the gluten content of food and beverages. Interestingly, a recent paper by Guerdrum and Bamforth[22] described the use of the competitive R5 ELISA method for measuring the level of gliadins in 28 commercial beers, however, this study did not assess the hordein proteins, only proteins containing the motifs specific for the R5 antibody.[32]

Mass spectrometry has been readily adopted for the characterization and quantification of proteins, in particular within the field of disease biomarker evaluation,[37] and has now begun to infiltrate the food and beverage industries. Intact gluten prolamins in food were first characterized and quantified using matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry.[38] Likewise, proteomic approaches using SDS-PAGE and RP-HPLC have been used to compare the proteins present in malts, worts and beers made from barley.[39] More recently, LC−MS/MS has been successfully applied to the study of gluten proteins in beer and this technology was proposed as a feasible alternative to ELISA-based methodologies.[40] MALDI-TOF mass spectrometry was applied to the identification of hordeins in barley and processed barley products.[41] A recent study utilized mass spectrometry for the detection of both peptide fragments and proteins present in beer in two commercial Italian barley malt beers.[23] No intact hordein proteins were detected, but a number of peptide fragments derived from γ3- and B-hordeins were present leading them to speculate that hordein are only present in trace amounts in beer.

The approaches described using MS as a tool to study gluten proteins have been global analyses, that is, they identify any proteins present in a given sample. MRM mass spectrometry[42] is a targeted approach that aims to identify and quantify a specific protein based on the peptides resulting from enzymatic digestion. MRM relies upon the inherent chemical and physical properties of the analyte, that is, its mass and its fragmentation pattern.

In this study, we have characterized the suite of prolamin proteins present in purified hordeins, wort and beer. We have implemented a database search strategy involving searching the complete spectral set against a translated EST database. We have demonstrated using tandem mass spectrometry that hordeins are indeed present in beer. Furthermore, we have performed relative quantification of the amount of four hordein protein families from three beers brewed from selectively bred barley lines and 60 commercial beers. The selectively bred barley line beers were brewed from grains of wild-type barley (Sloop) or of grains carrying mutations that prevent the accumulation of either B-hordeins (Risø 56) or C-hordeins (Risø 1508). We used scheduled MRM mass spectrometry, which is a fast, reliable, sensitive and specific method for quantification of gluten proteins within food and beverages.

EXPERIMENTAL METHODS

Plant Material

Barley line cv Sloop (wild type) was obtained from the Australian Winter Cereals Collection (Tamworth, Australia). Risø 56 (expressing no B-hordeins) and Risø 1508 (expressing no C-hordeins and decreased D- and B- hordeins)[43,44] were obtained from the Nordic Germplasm Bank (Alnarp, Sweden). Plants were grown in glasshouse conditions at 25 °C days and 20 °C nights and harvested seeds inspected to exclude contamination. For malting and brewing experiments cv Sloop, Risø 56 and Risø 1508 were grown side by side, at CSIRO Ginninderra Experiment Station, Canberra, in the field, and 10 kg of each harvested in December, 2007. The grains were malted by Barrett Burston Malting Co. Pty. Ltd., Richmond, Victoria, and 20 L batches of beer brewed by O'Brien Brewing, Ballarat, Victoria, using standard techniques.

Table 1. Mashing Protocol Used in the Production of Wort and Beer

Parameter	Sloop	Risø 56	Risø 1508
Volume (L)	15.0	18.6	14.1
Malt (kg)	3.60	4.00	3.33
Protein rest (Temp/time)	57 °C/ 20 min	54 °C/ 20 min	56 °C/ 20 min
Amylase rest (Temp/time)	65 °C/ 1 h	64−65 °C/ 1 h	63−65 °C/ 1 h
Original gravity (SG)	1.051	1.051	1.052
Final gravity (SG)	1.014	1.012	1.013
Alcohol (% vol)	4.8%	5.2%	5.1%

Prolamin Extraction

Prolamins in aqueous washed wholemeal flour (10 g) were dissolved in 50% (v/v) propan-2-ol, 1% (w/v) dithiothreitol (DTT), and precipitated with two volumes of propan-2-ol at −20 °C overnight. The precipitated prolamins were dissolved in 8 M urea, 1% DTT, 25 mM triethanolamine-HCl (pH 6), and purified by fast protein liquid chromatography (FPLC) on a 4 mL Resource RPC column (GE Healthcare, Sydney, NSW, Australia) eluted with a 30 mL linear gradient (at 2 mL/min) from 30% to 60% acetonitrile in 1% (v/v) trifluoroacetic acid (TFA).

Preparation of Wort and Beer

Barley was malted in a Joe White Micromalting System in several 800 g tins. The steeping regime involved: 8 h soaking, 9 h rest, 5 h soaking at 17 °C (Sloop); 8 h soaking, 10 h rest, 5 h soaking at 17 °C (Risø 56); and 7 h soaking, 8 h rest, 3 h soaking at 17 °C (Risø 1508). Germination occurred over 94 h at 16 °C for Sloop and 15 °C for the two hordein deletion mutants. The kiln program was over 21 h between 50 and 80 °C. The kilned malt was mashed as detailed in Table 1. After the indicated amylase rest time, the mash was bought to the boil and boiled for 1 h to produce the wort. During boiling, the boiling wort was bittered with Tettnang hops to achieve 21−22 IBUs. The wort was cooled overnight to 20 °C and then fermented with Fermentis US-05 yeast at 18−20 °C to completion after about 2 weeks. The unfiltered beer was kegged, and force carbonated before bottling.

Preparation of Beer

A selection of beers were collected (60 commercial beers as listed in Supplementary Table 1 alongside wild-type barley-derived beer and two hordein deletion beers) based on the stated ingredients or gluten content. Triplicate samples (1 mL) were taken from two different bottles and degassed to remove CO_2 under reduced pressure. Aliquots (100 μL) of degassed beer were taken and were reduced by addition of 20 μL of 50 mM DTT under N_2 for 30 min at 60 °C. To these solutions, 20 μL of 100 mM iodoacetamide (IAM) was added and the samples were incubated for 15 min at room temperature. To each solution 5 μL of 1 mg/mL trypsin (Sigma) or chymotrypsin (Sigma) was added and the samples incubated at 37 °C overnight. The digested peptide solution was acidified by addition of 10 μL of 5%

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formic acid and passed through a 10 kDa MW filter (Pall, Australia). The filtrate was lyophilized and reconstituted in 1% formic acid and stored at 4 °C until analysis.

Analysis of Undigested Wort and Beer

Wort and beer (0.1 mL) derived from the wild-type (Sloop) barley and hordein deletion mutant barley (Risø 56 and Risø 1508) were passed through a 10 kDa molecular weight cutoff filter (Pall) by centrifugation at 14000 rpm for 30 min to produce a peptide fraction amenable to LC−MS/MS. The peptide fraction (10 μL) was analyzed on the QStar Elite mass spectrometer.

Q-TOF MS

Samples were chromatographically separated on a Shimadzu nano HPLC system (Shimadzu Scientific, Rydalmere, Australia) using a Vydac MS C18 300 Å, column (150 mm × 0.3 mm) with a particle size of 5 μm (Grace Davison, Deerfield, MI) using a linear gradient of 2−42% solvent B over 20 min at a flow rate of 3 μL/min. The mobile phases consisted of solvent A (0.1% formic acid) and solvent B (0.1% formic acid/90% acetonitrile/10% water). A QStar Elite QqTOF mass spectrometer (Applied Biosystems) was used in standard MS/MS data-dependent acquisition mode with a nanoelectrospray ionization source. Survey MS spectra were collected (m/z 400−1800) for 1 s followed by three MS/MS measurements on the most intense parent ions (10 counts/second threshold, 2+ to 5+ charge state, and m/z 100−1600 mass range for MS/MS), using the manufacturer's "Smart Exit". Parent ions previously targeted were excluded from repetitive MS/MS acquisition for 30 s (mass tolerance of 100 mDa).

Linear Ion Trap (Triple Quadrupole) MS

Reduced and alkylated tryptic peptides were analyzed on an Applied Biosystems 4000 QTRAP mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a TurboV ionization source operated in positive ion mode. Samples were chromatographically separated on a Shimadzu Nexera UHPLC (Shimadzu) using a Phenomenex Kinetex C18 (2.1 mm × 10 cm) column with a linear gradient of 5−45% acetonitrile (ACN) over 15 min with a flow rate of 400 μL/min. The eluent from the HPLC was directly coupled to the mass spectrometer. Data were acquired and processed using Analyst 1.5 software. Information Dependent Acquisition (IDA) analyses were performed using an enhanced MS (EMS) scan over the mass range 350−1500 as the survey scan and triggered the acquisition of tandem mass spectra. The top two ions of charge state 2−5 that exceeded a defined threshold value (100000 counts) were selected and first subjected to an enhanced resolution (ER) scan prior to acquiring an enhanced product scan (EPI) over the mass range 125−1600.

Analysis of Mass Spectra and Database Searching

ProteinPilot 4.0 software (Applied Biosystems) with the Paragon Algorithm[45] was used for the identification of proteins. Tandem mass spectrometry data was searched against *in silico* tryptic or chymotryptic digests of Triticeae proteins of the Uniprot (version 2011/05) and NCBI (version 2011/05) databases. All search parameters were defined as iodoacetamide modified with cysteine alkylation, with either trypsin or chymotrypsin as the digestion enzyme. Modifications were set to the "generic workup" and "biological" modification sets provided with this software package, which consisted of 126 possible modifications, for example, acetylation, methylation and phosphorylation. The generic workup modifications set contains 51 potential modifications that may occur as a result of sample handling, for example, oxidation, dehydration and deamidation. Peptides with one missed cleavage were included in the analysis. The False Discovery Rate (FDR) was calculated by the PSPEP algorithm[46] embedded within ProteinPilot, and only protein identifications that had a ProteinPilot confidence score of 99% or better (estimated global FDR of 1% or lower; Unused Score above 2.0) were accepted.

Construction and Application of a Custom-built Cereal Database

A nonredundant custom cereal seed storage protein database was constructed by including all reported protein sequences from nucleotide entries in NCBI, TIGR Gene Indices, or TIGR Plant Transcript Assemblies belonging to *Triticum*, *Hordeum*, *Avena*, *Secale* and *Triticosecale* species. The nucleotide sequences, for the above species, were translated in six frames, trimmed to keep only the longest open reading frame. The resulting protein sequence set was then made nonredundant. Only sequences with 100% match from start to finish were collapsed together, to maintain all variations. Lastly, these files were filtered to retain only entries containing the words gluten, gliadin, glutenin, hordein, avenin or secalin. Tandem mass spectrometry data was searched against the custom cereal database.

Protein Alignment and Identification of Prototypic Peptides

All known hordein proteins in the Uniprot database and predicted hordein proteins in the TIGR database were aligned. Within each family (B, C, D or γ), peptides that were common were selected as representative of the family. MRM transitions were determined for each peptide where the precursor ion (Q1) m/z was based on the size and expected charge and the fragment ion (Q3) m/z values were predicted using known fragmentation patterns and/or the data collected in the characterization workflows. Up to six transitions were used in the preliminary analyses and the MRM transitions were refined and the top two MRM transitions were selected per peptide for use in the final method, wherein the most intense MRM transition was used as a quantifier and the second most intense transition was used as a qualifier.

MRM Mass Spectrometry

MRM experiments were used for quantification of the hordein-derived tryptic peptides. For both IDA- and MRM-triggered MS/MS experiments, the scan speed was set to 1000 Da/s and peptides were fragmented in the collision cell with nitrogen gas using rolling collision energy dependent on the size and charge of the precursor ion. Quantification of hordein peptides was achieved using scheduled MRM scanning experiments (Figure 2A) using a 120 s detection window for each MRM transition and a 1s cycle time.

Relative Quantification of Hordeins

The relative quantification of each hordein was performed by integrating the peak area of the most intense MRM transition for each peptide. The average peak area was determined by taking the mean of two replicate injections (on different days) from bottles A and B (representing the biological replicates). The results are presented as the percentage of each hordein protein relative to the average hordein content of all gluten-containing beers.

RESULTS AND DISCUSSION

Characterization of Enriched Hordein Fraction

Hordeins extracted from flour were purified by FPLC. The purified hordein fractions were reduced, alkylated and subjected to both trypsin and chymotrypsin digestion. Following enzymatic digestion, the sub-10 kDa fraction was analyzed by LC−MS/MS

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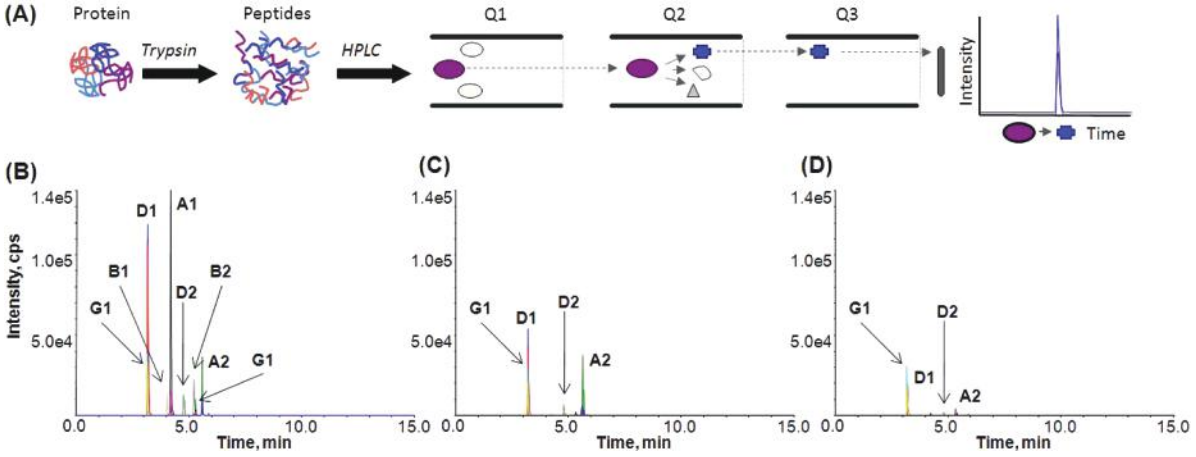

Figure 2. Hordeins identified in beer derived from barley flour using multiple reaction monitoring (MRM). MRM is a useful tool for the quantification of peptides and proteins. (A) Following tryptic digestion of a protein, the proteolytic fragments are chromatographically separated by HPLC and analyzed by MRM mass spectrometry. The first quadrupole (Q1) selects the first peptide m/z value (precursor mass) and transmits this ion to the collision cell (Q2). Collision-induced dissociation results in the production of fragment ion series relating to the amino-acid sequence of the proteolytic fragments. Diagnostic fragment ions are then selected in the third quadrupole (Q3) and transmitted to the detector allowing quantification of the peptides of interest. Typically three MRM transitions per peptide are used and at least two peptides per protein. (B) MRM analysis of beer derived from wild-type barley (Sloop). Each labeled peak represents a peptide derived from a tryptic digest of the hordein protein families: A1-A2 are peptides derived from the avenin-like A proteins; B1−B2 are B-hordein peptides; D1-D2 are D-hordein peptides; and G1-G2 are γ-hordein peptides. Beer brewed from (C) Risø 56 barley and (D) Risø 1508 barley are also shown. Each MRM analysis is presented on the same y-axis scale.

to identify the hordeins present in a purified prolamin fraction from flour to provide the complete suite of hordein proteins that might be expected to be found in beer brewed from this flour. Using a 1% FDR, a total of 144 proteins were identified after trypsin digestion and a total of 55 proteins were identified after chymotrypsin digestion. Table 2A lists the hordein protein products detected following tryptic digestion. The data is available in the PRIDE database[47] (www.ebi.ac.uk/pride) under accession numbers 19482−19483. The data was converted using PRIDE Converter[48] (http://pride-converter.googlecode.com).

Among the most abundant proteins detected were the previously reported B3-hordein[49] (P06471), γ-3-hordein[50] (P80198) and the predicted γ-1-hordein[51] (P17990). Likewise, the D-hordein[52] (Q84LE9) was detected in abundance. A number of lesser abundant proteins were detected including two γ-hordeins and a B1-hordein that were not present in either the NCBI or Uniprot databases (Table 2A). These proteins were identified by searching a custom-built database comprising translated cereal proteins from nucleotide entries in NCBI, TIGR Gene Indices, or TIGR Plant Transcript Assemblies. Several peptides matching to predicted γ-gliadins and glutenins (from wheat) and avenin-like protein-A (from wheat and goat-grass) were detected. The avenin-like A proteins were recently reported to be present in beer[23] based on the detection of a 15 amino acid peptide (QQCCQPLAQISEQAR) resulting from tryptic digestion of a 16−17 kDa protein band isolated by SDS-PAGE. In this study, we identified the same peptide along with an additional 13 aa peptide matching to the same predicted protein sequence (F2EGD5). An 11 aa (MVLQTLPSMCR) peptide mapping to an avenin-like A protein (Q2A782) from *Aegilops cylindrica* (jointed goatgrass) was also detected, but no orthologous protein in barley could be found in the public protein databases. A subsequent search of the translated EST database (TIGR) revealed a *H. vulgare* protein that explained the 11 aa peptide. Homology between the predicted avenin-like protein-A and γ-hordein proteins sparked our interest in this family of barley proteins and these were included in subsequent analyses. The alignment of the identified avenin-like A proteins from *Aegilops cylindrica* and *Hordeum vulgare* is given in Supplementary Figure 1 (Supporting Information).

Single peptide identifications hinted at the presence of C-hordeins within the hordein fraction, however, sequence alignment of known C-hordein proteins revealed an absence of tryptic cleavage sites within these glutamine-rich proteins. Consequently, chymotryptic digest of the hordein fraction yielded identification of C-hordeins with up to 80% sequence coverage (Table 2B), highlighting the need for an alternative digestion strategy to characterize this class of hordeins.

Characterization of Wort and Beer

Analysis of wort, the liquid extracted from the mashing process during brewing, and beer were then conducted and a similar suite of prolamins were identified (Table 3A, B). A total of 27 proteins were identified in wort and 79 in beer, with the most abundant proteins being nonspecific lipid transfer protein 1 (LTP1) and the α-amylase trypsin inhibitors (CMd, CMb, CMa). The complete list of proteins identified is given in Supplementary Table 2 (Supporting Information). The gluten proteins identified in wort included the avenin-like A protein (18 peptides), γ-hordein-3 (10 peptides) and D-hordein (4 peptides) that were previously observed in the enriched hordein fraction. It was interesting to note, however, that >50% of the peptides were semitryptic (cleaved at one end at a site other than Lys/Arg), suggesting that significant degradation of the proteins had occurred during the brewing process. The avenin-like A protein (GenBank: BE195337) identified from the EST database search was detected with >60% sequence coverage (12 peptides) increasing the confidence of this protein identification (Table 3B).

C-hordein proteins were noticeably absent in beer, but to ensure this was not a false positive owing to the low number of tryptic sites, we performed a chymotryptic digest which affirmed the absence of C-hordeins in beer (Table 3C). A similar strategy

Table 2. Prolamin Proteins Identified in FPLC Purified Fraction of Barley Flour

A. Prolamins identified in flour (trypsin digestion)

Uniprot accession	NCBI accession	TIGR accession	name	score	coverage	peptides
		BE454297	B3-hordein[a]	57.03	87.1	52
P80198	1708280	TA29416_4513	γ-hordein-3	38.40	54.0	32
Q84LE9	75147012	TA30219_4513	D-hordein[b]	34.70	55.6	41
P17990	123464		γ-hordein-1	27.70	74.7	23
		TA30139_4513	γ-hordein[a]	18.95	45.5	24
Q40026	75220903	TA29493_4513	B1-hordein[b]	8.58	46.6	19
Q4G3S1	122220129		B3-hordein[c]	5.55	15.7	10
P06472	123460		C-hordein[c]	4.56	23.9	4
C7FB16	255348358		B-hordein[b]	4.00	22.4	22
Q2A782	122238432		Avenin-like A[c]	4.00	14.4	2
F2XAR6	327365751		γ-gliadin[b]	3.89	9.5	3
		TA29452_4513	B1-hordein[a]	3.87	52.2	18
P17991	123461		C-hordein[c]	3.87	95.8	3
		AJ433315	γ-hordein[a]	3.53	99.1	19
P06471	123459	HVB3HORD	B3-hordein	2.00	73.1	42
B9VSH7	222538169		D-hordein (similar to HMW glutenin)[b]	2.00	16.1	9
Q3YAF9	122217636		B-hordein[b]	2.00	21.7	8
B5A818			B-hordein (similar to LMW glutenin)[b]	2.00	27.2	7
D4HNB5			B-hordein (similar to LMW glutenin)[b]	2.00	13.2	4
Q8S3W0	75159492		D-hordein (similar to HMW glutenin)[b]	2.00	5.0	6
F2EGD5	326501830		Avenin-like A[c]	2.00	16.2	2

B. Prolamins identified in flour (chymotrypsin digestion)

Uniprot accession	NCBI accession	TIGR accession	name	score	coverage	peptides
P06471	123459	HVB3HORD	B3-hordein	85.95	86.4	51
Q84LE9	75147012	TA30219_4513	D-hordein[b]	54.28	36.2	28
Q41210	75102504		C-hordein[b]	50.10	88.7	44
P80198	1708280	TA29416_4513	γ -hordein-3	36.38	69.9	19
Q40053	19001		C-hordein (Hor1−17)[b]	28.93	83.9	41
		TA30139_4153	γ −hordein[a]	21.31	59.2	11
P06470	123458		B1-hordein	10.13	25.1	14
Q0PIV6	110832715		B-hordein[b]	10.00	62.4	22
P17990	123464		γ -hordein-1[c]	8.00	50.9	7
P17992	123462		C-hordein[c]	6.02	65.1	7
Q571R2	75271341		C-hordein (similar to σ-gliadin)[c]	4.00	61.3	3
		TA29452_4513	B1-hordein[a]	2.13	59.4	14
C7FB16	255348358		B-hordein[b]	2.02	61.9	15
		TA28105_4153	Similar to C-hordein[a]	2.01	53.4	1
		BG416634_F0	B3-hordein[a]	2.00	81.5	32
		BI950745_F1	B3-hordein[a]	2.00	33.6	15
		TA29459_4513	B-hordein (similar to GBSS Wx-TmA)[a]	2.00	60.9	12
Q5PU42	56126405		B-hordein (similar to LMW glutenin)[b]	2.00	22.6	6
Q4G3S5	57118089		B3-hordein[c]	2.00	24.0	6

[a] Novel protein identified in database search against unannotated TIGR ESTs. [b] Protein identification supporting genome-derived predicted protein (Uniprot). [c] Protein identification supporting annotated transcript evidence (Uniprot).

involving cocktail digestion (combined trypsin and chymotrypsin) of gluten proteins was employed recently.[53] The absence of C-hordeins is presumably owing to their insolubility in water. C-hordeins consist of multiple octapeptide repeats with a consensus sequence of PQQPFPQQ rendering them highly insoluble. It has previously been shown by ELISA that the majority of B- and C-hordeins are lost during the brewing process.[54] We have shown that a large number of C-hordein degradation products are identified in wort, but that the majority of these peptides do not survive the brewing and filtration steps leading to the production of beer.

It is known that many proteins are degraded and/or modified during the brewing process. In order to characterize peptide fragments, the wort and beer were passed through a 10 kDa

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Table 3. Prolamin Proteins Identified in Wort and Beer

A. Prolamins identified in wort (trypsin digestion)						
Uniprot accession	NCBI accession	TIGR accession	name	score	coverage	peptides
F2EGD5	326501830		Avenin-like A[a]	29.33	41.6	18
P80198	1708280	TA29416_4513	γ-hordein-3	18.05	33.2	10
		BE195337	Avenin-like A[b]	14.00	47.0	9
Q84LE9	75147012	TA30219_4513	D-hordein[c]	8.08	8.9	4
B. Prolamins identified in beer (trypsin digestion)						
Uniprot accession	NCBI accession	TIGR accession	name	score	coverage	peptides
P80198	1708280	TA29416_4513	γ-hordein-3	55.62	53.6	33
Q84LE9	75147012	TA30219_4513	D-hordein[c]	30.16	22.2	23
P06471	18914	HVB3HORD	B3-hordein	17.81	24.6	9
P17990	123464		γ-hordein-1[a]	16.98	31.9	9
		BE195337	Avenin-like A[b]	15.66	64.6	12
F2EGD5	326501830		Avenin-like A[a]	8.06	35.3	8
Q4G3S5	57118089		B3-hordein[a]	5.48	12.6	6
Q40026	75220903	TA29493_4513	B1-hordein[c]	2.01	8.6	7
Q94IL5	75250230		D-hordein (similar to HMW glutenin)[c]	2.00	4.6	8
C. Prolamins identified in beer (chymotryptic digestion)						
Uniprot accession	NCBI accession	TIGR accession	name	score	coverage	peptides
P80198	1708280	TA29416_4513	γ-hordein-3	19.75	56.1	12
Q40022	829269		B1-hordein[a]	13.80	51.4	9
Q84LE9	75147012	TA30219_4513	D-hordein[c]	12.00	26.0	6
P17990	123464		γ-hordein-1[a]	5.59	22.5	3
F5A7G6			B-hordein (similar to LMW glutenin)[c]	2.00	23.2	5
Q6EEZ0			γ-hordein-3[c]	2.00	25.8	2

[a] Protein identification supporting annotated transcript evidence (Uniprot). [b] Novel protein identified in database search against unannotated TIGR ESTs. [c] Protein identification supporting genome-derived predicted protein (Uniprot).

molecular weight cutoff filter and analyzed without enzymatic digestion. 1D-PAGE analysis revealed that the filtration step was efficient in removing proteins from beer (Supplementary Figure 2, Supporting Information). MS analysis revealed the presence of several truncated or degraded hordein products. Table 4 lists the proteins detected in wort and beer and Supplementary Table 3 (Supporting Information) lists the peptide fragments identified. In addition to hordein peptides, peptides derived from serpin-Z4, nonspecific lipid transfer protein 1, α-amylase, β-amylase, hordoindoles (B1, B2) and GAPDH were identified in beer (Supplementary Table 2, Supporting Information). Of interest, was the large number of C-hordein fragments observed in wort with only trace levels of C-hordein peptides detected in beer. The characterization of beer in the absence of enzymatic digestion clearly demonstrates that in addition to intact hordeins, a large number of partially degraded hordein fragments are present and these may also contribute to coeliac toxicity. Many of these peptide fragments contained runs of Gln and Pro that may elicit an immunological response in coeliacs. Examples of potential immunogenic peptides detected are FVQPQQQPFPLQPHQP (avenin-like A; GenBank: TA31086), YPEQPQQPFPWQQPT (γ-1-hordein; P17990), LERPQQLFPQWQPLPQQPP (γ-3-hordein; P80198) and LIIPQQPQQPFPLQPHQP (C-hordein; P17991), where the underlined sequence bears high homology with immunogenic peptides reported previously.[3,32]

Relative Quantification of Hordeins in Beer by MRM Mass Spectrometry

The proteomic characterization of purified hordeins and beer enabled the elucidation of the major hordein proteins present in barley. From each of the protein families, a single isoform was selected to monitor the gluten content of three beers brewed from selectively bred barley lines. Multiple tryptic peptides (≥2 peptides/protein) were selected for development of a quantitative assay. Where these peptides had previously been detected in the discovery experiments, the peptide m/z and fragment ion information was used to determine the MRM transition to be used. A number of peptides that were not initially identified were included in the MRM assay so that a minimum of two peptides per protein were used. In these instances, the retention times were not known and the MRM transitions could not be scheduled in the first pass experiments. The peptide retention times were determined and subsequent experiments used scheduled MRM transitions. Beer derived from a single elite Australian malting barley ("Sloop") was used for the development and refinement of the MRM method as it contained the full complement of hordein proteins, whereas the barley variants (Risø 56 and Risø 1508) were expected to be low in, or devoid of, B- and C-hordeins. Figure 2 shows the analysis of the wild-type and two hordein deletion beers using MRM analysis. The eight selected peptides (three MRM transitions per peptide) are all clearly observable for wild-type barley beer (Figure 2B). The Risø 56

Table 4. Prolamin Protein Fragments Identified in Wort and Beer

A. Prolamins identified in wort (no digestion)

Uniprot accession	NCBI accession	TIGR accession	name	score	coverage	peptides
Q84LE9	75147012	TA30219_4513	D-hordein[a]	50.91	47.6	27
Q40053	19001		C-hordein (Hor1−17)[a]	17.78	68.5	8
P06470	123458		B1-hordein	17.65	32.4	9
		TA30139_4153	γ-hordein[b]	16.62	36.4	8
P80198	1708280	TA29416_4513	γ-hordein-3	16.31	37.7	8
Q41210	442524		C-hordein[a]	6.57	72.3	9
Q571R2	75271341		C-hordein (similar to σ-gliadin)[c]	6.26	34.4	4
Q40026	75220903	TA29493_4513	B1-hordein[a]	6.19	29.0	5
		TA29459_4513_F2	B-hordein (similar to GBSS Wx-TmA)[b]	4.10	61.7	2
P17991	123461		C-hordein[c]	4.05	80.6	6
Q2QL53	122202965		α-gliadin[a]	4.03	9.3	3
P06471	18914	HVB3HORD	B3-hordein	3.48	27.7	3
C4NFP4			σ-secalin[a]	2.05	57.4	2
Q40055	75220910		C-hordein[a]	2.00	57.6	4
B6UKV5	209972037		γ-gliadin[a]	2.00	17.9	2
		BE195337	Avenin-like A[b]	2.00	6.7	1

B. Prolamins identified in beer (no digestion)

Uniprot accession	NCBI accession	TIGR accession	name	score	coverage	peptides
Q84LE9	75147012	TA30219_4513	D-hordein[a]	22.24	61.6	11
P80198	1708280	TA29416_4513	γ -hordein-3	14.10	55.7	7
P17990	123464		γ-hordein-1[c]	8.04	53.3	4
P17991	123461		C-hordein[c]	3.40	66.0	2
		BE195337	Avenin-like A[b]	3.30	39.6	2
P06471	18914	HVB3HORD	B3-hordein	3.01	47.4	1

[a] Protein identification supporting genome-derived predicted protein (Uniprot). [b] Novel protein identified in database search against unannotated TIGR ESTs. [c] Protein identification supporting annotated transcript evidence (Uniprot).

beer shows an approximate 3-fold decrease in the amount of the D-hordein peptides and the B-hordein peptides are noticeably absent (Figure 2C). The Risø 1508 beer showed a further decrease in the amount of each peptide measured, but a trace amount of avenin-like A protein (labeled A1) can be observed (Figure 2D).

The reproducibility of the analytical method was assessed by examining the single cultivar barley beer ("Sloop"). First, the beer was subjected to multiple freeze−thaw cycles (either 1, 10, 20, or 50 cycles) and no significant change (coefficient of variation, CV, of <15%) was observed in the peak area for each of the monitored MRM transitions even after 50 cycles (Supplementary Table 4, Supplementary Figure 3A, Supporting Information). Second, the digestion efficiency was examined by six replicate digestions yielding a CV of <15% (Supplementary Table 4, Supplementary Figure 3B, Supporting Information). The analytical reproducibility was assessed by performing four replicate injections of each digest with a CV of <15%. Finally, the variation in hordein content between two different bottles of beer was assessed and found to be <10% (Supplementary Table 4, Supplementary Figure 3B, Supporting Information).

Analysis of Commercial Beer

Further validation of this MRM approach was provided by analyzing the gluten content of a selection of 60 commercial beers, including low gluten and gluten-free beers. Duplicate samples (from separate bottles) were treated by reduction, alkylation and digestion and were analyzed by MRM mass spectrometry. Figure 3 shows the relative quantification of the avenin-like A proteins (A), B-hordeins (B, C), D-hordeins (D) and γ-hordeins (E). The average hordein content of all commercial beers (excluding gluten-free beers) were calculated and the individual hordein content of each beer were compared to the average (Supplementary Table 1, Supporting Information). The commercial beers were observed to vary in the type (hordein families present) and the amount (between 1 and 380% cf. average for any given hordein protein). Eight of the beers (17, 47, 49, 50, 51, 52, 58 and 60) were labeled as gluten-free as they were brewed from sorghum malt, teff, rice, millet or maize. These cereals lack the gluten proteins problematic in barley and wheat. The MRM assay confirmed that they were devoid of the hordein proteins targeted. Beers 17 and 50−52 were sorghum-based, beer 47 did not specify what it is made from, beer 49 was millet-based, beer 58 was brewed from sorghum malt, teff and rice and beer 60 was a non-cereal derived beer. In the examination of two beers (57 and 59) that have been classified as low-gluten (<10 ppm), the relative hordein content was not dissimilar to the average hordein content across the range of beers tested. Beer 57 showed low avenin-like A protein levels (∼50% cf. average), but surprisingly showed significant levels of peptides derived from the B1- (>300% cf. average), D- (∼105%) and γ3-hordeins (∼62%). Beer 59, showed low, but significant levels of B1-, D- and γ-hordeins (55%, 42%

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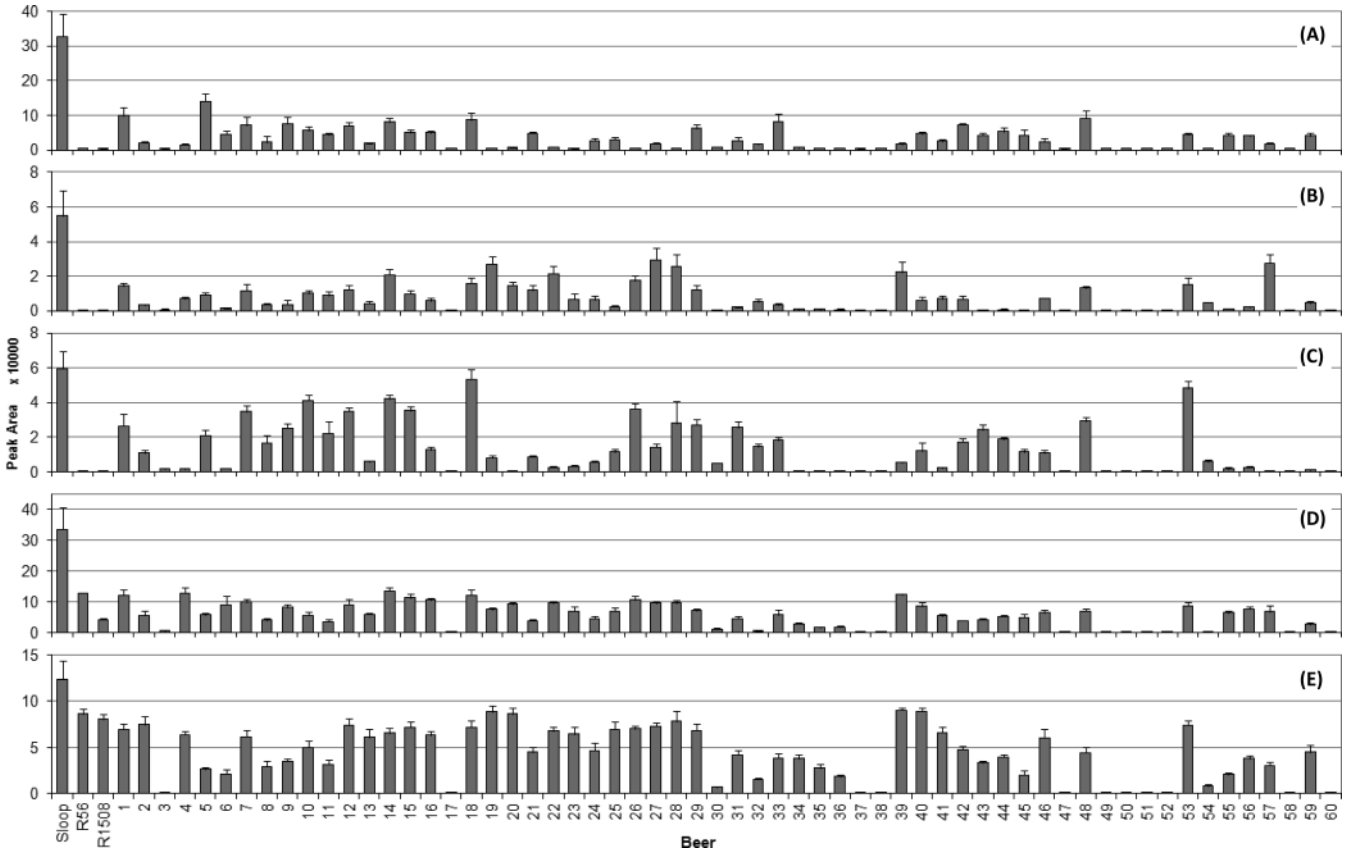

Figure 3. Relative hordein quantification in beers. The peak area of selected peptides representing the most abundant hordeins detected in beer: (A) QQCCQPLAQISEQAR representing avenin-like A protein; (B) VFLQQQCSPVR representing B1-hordein; (C) VFLQQQCSPVPMPQR representing B3-hordein; (D) ELQESSLEACR representing D-hordein; and (E) QQCCQQLANINEQSR representing γ-hordein-3. These representative peptides have been used to illustrate the relative amount of the major hordein proteins in wild-type (Sloop) and three hordein deletion beers (Risø 56 and Risø 1508 respectively) and in 60 commercial beers. The small peak area seen for the gluten free beers 17, 47, 49−52, 58, and 60 was due to a low level of noise in the signal, and not due to detection of significant levels of hordein.

and 92% respectively) and equivalent levels of the avenin-like A protein to those observed in the gluten-containing beers.

A number of the tested beers, despite not having a defined gluten-status, showed lower than average gluten content. For example, beers 3, 30, 37, and 38 showed very low levels (<5%) of avenin-like A, B1-hordein and G3-hordein and low, but detectable levels of B3- (∼11%) and D-hordeins (∼10%). Beer 54 was devoid of avenin-like A proteins and D-hordeins, but showed low levels of B-hordeins (40−60%) and γ-hordeins (∼16%). The beers that showed levels of hordein were also subjected to global proteomic profiling to test if any other forms of gluten (gliadin, glutenin or hordein) were present. Using this analytical procedure we were unable to detect gluten proteins in the gluten-free beers (17, 47, 49−52, 58 and 60). Trace levels of D-hordein (Q84LE9) were detected in beers 3, 37, and 38 with beer 38 also containing a B-hordein (Q40026). While no hordein proteins were detected in beer 30, a γ-gliadin (Q9XEW0) was detected.

CONCLUSIONS

Coeliac disease is exacerbated by the intake of prolamins present in wheat, rye, barley and in some coeliacs, oats. The only treatment for CD is a life-long gluten-free diet. Many food products have entered the market suitable for CD patients, who make up ∼1% of the population. Beers contain gluten derived from grains used in the brewing process.

The gluten level in beer may be measured using ELISA, however, there are many limitations associated with accurate measurement of hordeins using current ELISA technology. In this study, we have implemented a mass spectrometric assay to first characterize the complete suite of hordeins in purified hordein preparations, wort and beer and second to perform relative quantification of the most abundant hordein proteins. We have developed a robust and sensitive quantification methodology for the measurement of hordein (gluten) in beer.

Analysis of hordein deletion beers and commercially available beers confirmed that all the barley based beers tested contained hordeins, while no hordeins were detected in the gluten-free beers analyzed. Significantly both barley based low-gluten beers tested, in which the hordein concentration is reduced by proprietary processing steps during brewing to reduce the concentration in the final beer product, had substantial levels of one or more hordein proteins.

ASSOCIATED CONTENT

Supporting Information

Additional information about identification of proteins and the beers tested in this study. Supplementary Figures showing alignment of avenin-like A proteins and demonstrating reproducibility of the analytical method are also provided. This

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

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