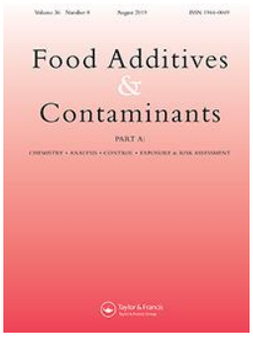

Food Additives & Contaminants: Part A

ISSN: 1944-0049 (Print) 1944-0057 (Online) Journal homepage: https://www.tandfonline.com/loi/tfac20

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To cite this article: Katherine L. Fiedler, Wanying Cao, Liyun Zhang, Magdalena Naziemiec, Binaifer Bedford, Lanlan Yin, Nicholas Smith, Matthew Arbuckle, Arnoldo Lopez-Hernandez & Lauren S. Jackson (2019) Detection of gluten in a pilot-scale barley-based beer produced with and without a prolyl endopeptidase enzyme, Food Additives & Contaminants: Part A, 36:8, 1151-1162, DOI: 10.1080/19440049.2019.1616830

To link to this article: https://doi.org/10.1080/19440049.2019.1616830

FOOD ADDITIVES & CONTAMINANTS: PART A
2019, VOL. 36, NO. 8, 1151–1162
https://doi.org/10.1080/19440049.2019.1616830

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Detection of gluten in a pilot-scale barley-based beer produced with and without a prolyl endopeptidase enzyme

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ABSTRACT

Immunochemical and mass spectrometric methods were used to examine the gluten composition of a gluten-reduced beer produced by brewing with barley malt in the presence of prolyl endopeptidase (PEP) and a final filtration treatment with diatomaceous earth and perlite. The competitive ELISA is generally considered appropriate for the analysis of hydrolysed gluten, but it is not considered a scientifically valid method for the quantification of gluten in fermented or hydrolysed foods due to the lack of an appropriate reference standard. As no single analytical method can capture the spectrum of gluten-derived products in beer, a comprehensive approach was employed to analyse the intact and hydrolysed fractions of gluten with complementary methods. The combination of PEP addition and diatomaceous earth/perlite filtration was more effective at reducing the concentration of detectable gluten than each of the treatments alone. However, gluten proteins and/or polypeptides were observed in filtered, PEP-treated beers using sandwich ELISA methods, western blot, and bottom-up mass spectrometry. In addition, mass spectrometry results showed that the number of hydrolysed gluten peptides was almost unaffected by the filtration process. Gluten peptides that contained potentially immunopathogenic sequences were identified in the filtered PEP-containing beers by MS. Variability in gluten composition was observed between three replicate pilot-scale productions, suggesting that the gluten profile in beer could differ from batch to batch. As there is uncertainty in the detection and quantification of gluten in hydrolysed and fermented foods, characterisation of hydrolysed gluten by complementary analytical methodologies is recommended.

ARTICLE HISTORY
Received 13 February 2019
Accepted 25 April 2019

KEYWORDS
Hydrolysed gluten; PEP; filtration; quantification; mass spectrometry; immunopathogenic sequence; celiac disease; hordein; gluten-reduced; fermentation

Introduction

Gluten refers to a complex class of storage proteins present in the endosperm of wheat, barley, rye, and their crossbred varieties (Koehler et al. 2014). Gluten comprises two protein fractions: monomeric prolamins (specifically, gliadin in wheat, hordein in barley, and secalin in rye) and polymeric glutelins. In genetically susceptible individuals, gluten-derived peptides that cross the gut lumen can trigger an autoimmune response known as celiac disease (CD), which is characterised by inflammation and subsequent damage to the small intestine (Green and Cellier 2007). As the only treatment for CD is strict avoidance of dietary gluten, analytical methods that can reliably detect and quantify gluten in food are essential.

The amount of gluten in beer brewed from gluten-containing grains depends on the various stages of the brewing process: grain malting, mashing, lautering, clarification, fermentation, and finishing treatments such as filtration (Watson et al. 2018). The gluten content of beer can also be affected by the addition of enzymes, such as prolyl endopeptidase (PEP), during the brewing process. PEP, which cleaves C-terminally to proline residues, is sometimes used to dehaze and stabilise beer by removal of proline-rich proteins and polypeptides like gluten (Asano et al. 1982; Bamforth 1999; Stepniak et al. 2006). Beer is often filtered before bottling to remove yeast and colloidal particles, which increases clarity and provides microbiological stability

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(Mermelstein 1998). Filtration is also used by some in the brewing industry to reduce the gluten concentration of beer (Taylor et al. 2015; Watson et al. 2019).

Enzyme-linked immunosorbent assay (ELISA) is the current gold standard method for the detection and quantification of gluten, even though the ability of any particular ELISA kit to accurately determine true gluten content is under debate (Diaz-Amigo and Popping 2013). Different gluten-specific ELISAs provide various quantitative results for the same sample due to differences in antibodies, extraction procedures, reference standards, and kit format (Diaz-Amigo and Popping 2012; Rzychon et al. 2017). The Morinaga Wheat/Gluten (Gliadin) and RIDASCREEN® Gliadin (abbreviated R5 sand), both sandwich ELISAs, are currently used by the U.S. FDA in its enforcement of gluten-free food labelling. However, sandwich-based ELISA methods are not able to accurately quantify gluten in fermented and hydrolysed foods, such as beer (U.S. Food and Drug Administration [FDA] 2015). Sandwich ELISAs require two epitopes for detection and are only appropriate for the analysis of intact gluten. Competitive-based ELISA methods, such as the RIDASCREEN® Gliadin Competitive (abbreviated R5 comp), require a single epitope for detection and are better suited for the analysis of hydrolysed gluten. However, the R5 comp is not considered scientifically valid for the quantification of gluten in fermented or hydrolysed foods due to the lack of an appropriate reference standard (Revised Interim Policy on Gluten Content Statements in the Labeling and Advertising of Wine, Distilled Spirits, and Malt Beverages 2014; Food Labeling; Gluten-Free Labeling of Fermented or Hydrolysed Foods 2015; Panda et al. 2015a; Colgrave et al. 2017; Kerpes et al. 2017).

The accurate quantification of gluten by ELISA depends on a reference material that properly represents the type of gluten proteins in a sample because the kit antibodies have different affinities for wheat-, rye-, and barley-derived gluten (Tanner et al. 2013). For example, the Morinaga kit uses a polyclonal antibody raised against wheat which only shows 41.6% specificity for barley (Sharma et al. 2015). Commercial ELISA antibodies also have different selectivities for the various gluten protein types within a cereal grain. For example, the RIDASCREEN® methods use the R5 monoclonal antibody (raised against ω-secalins with strong cross-reactivity to wheat gliadin) which has lower affinity for D-hordeins and higher affinity for C-hordeins (Lexhaller et al. 2017). Unfortunately, no reference material is available for hydrolysed barley gluten produced during the brewing process or PEP treatment. The Morinaga kit employs a wheat protein standard and the R5 sand uses a gliadin reference material extracted from a mixture of 28 European wheat cultivars (van Eckert et al. 2006). The R5 comp uses a mixture of wheat, rye, and barley prolamins that have been digested with pepsin and trypsin to mimic hydrolysis; however, the hydrolysis products obtained from the pepsin/trypsin digest do not accurately represent those generated during yeast fermentation and PEP treatment (Panda et al. 2015a).

Mass spectrometry is a complementary proteomics technique that can be used for the detection and characterisation of gluten. A typical bottom-up mass spectrometry method involves the digestion of isolated proteins with a well-characterised enzyme, such as trypsin or chymotrypsin (Aebersold and Goodlett 2001). The resultant peptides are then analysed via liquid chromatography-tandem mass spectrometry (LC-MS/MS) where they are separated on a reversed-phase column before introduction into a mass spectrometer and subsequent fragmentation. The spectra acquired, which include the mass of the peptides and their respective fragment ions, are searched against a database of known protein sequences using bioinformatics to produce a list of identified peptides and inferred proteins (Aebersold and Mann 2003).

Several groups have utilised mass spectrometry to detect gluten in gluten-reduced beers that have tested below the limit of quantification for gluten by ELISA (Real et al. 2014; Panda et al. 2015a; Akeroyd et al. 2016; Knorr et al. 2016; Colgrave et al. 2017; Di Ghionno et al. 2017; Liao et al. 2017; Fiedler et al. 2018). A wheat gluten-incurred, sorghum beer brewed with and without the addition of PEP has been used previously as a model to study the effects of PEP on gluten

(Panda et al. 2015a). A two-pronged approach, which separately isolated hydrolysed gluten peptides with a molecular weight cut off filter and precipitated intact gluten proteins with acetone before LC-MS/MS analysis, demonstrated that PEP did not cleave at every proline residue and intact, or partially intact, gluten proteins were present in PEP treated beer (Fiedler et al. 2018). In addition, some of the gluten observed in the PEP-treated beer contained potentially immunopathogenic sequences, which are amino acid sequences capable of eliciting CD. However, this model did not involve the mashing of malted gluten-containing grains which is an important stage of the brewing process that dictates the gluten profile at the start of fermentation and before PEP treatment. Missed PEP cleavage sites (P-X motifs) and gluten peptides derived from hydrolysed hordeins, some larger than 30 kDa in size, were also detected in commercial PEP-treated barley beers by size-based protein fractionation followed by LC-MS/MS (Colgrave et al. 2017). However, as noted by the authors, the analysis was performed on only one batch of each beer and details on the manufacture of each commercial beer were not fully known. The analysis of barley-based beers for immunopathogenic sequences is limited by the fact that few of the known epitopes are derived from barley (Sollid et al. 2012; Goodman et al. 2016). Even though previous reports have suggested that PEP degrades all known immunopathogenic sequences (Akeroyd et al. 2016), Colgrave *et al.* observed some homology between wheat-derived epitopes and several gluten peptides found in commercial barley-based, PEP-treated beers (Colgrave et al. 2017).

A combination of gluten-minimisation techniques can be used to maximise the removal of gluten from beers produced from gluten-containing grains, according to ELISA analysis (Watson et al. 2019). However, the true gluten content of beer cannot be determined since there is uncertainty in interpreting competitive ELISA results for the quantification of hydrolysed gluten in terms of equivalent amounts of intact gluten. Therefore, the safety of gluten-reduced beers for individuals with CD remains controversial. A recent study has shown that sera from a subset of active-CD patients reacted to the gluten in a commercially available gluten-reduced beer (Allred et al. 2017). In this work, immunochemical and mass spectrometric methods were used in complement to provide a comprehensive characterisation of the effects of PEP addition rate and a final filtration step with a mixture of diatomaceous earth and perlite (effective pore size of 0.45 μm) on detectable gluten in beer brewed with barley malt on a pilot-scale.

Materials and methods

Beer production

A pilot-scale brewing plant located in the University of Wisconsin-Madison (UW-Madison) Department of Food Science was used to produce three independent brew trials of barley-malt beer. Brewing ingredients that were used in this study included Barley Brewers Malt #5298 (Briess; Chilton, WI), Cascade hops (Brew and Grow; Crystal, IL), and Wyeast #1272 American Ale II (Wyeast Lab; Odell, OR). PEP (Brewers Clarex®; activity ≥5.0 Proline Protease Units (PPU)/g) was provided by DSM Food Specialties (Netherlands). In each trial, wort (55 L) was produced with barley malt (28.3 kg), water (9.3 kg), and hops (28 g) following the brewing process illustrated in Supplementary Figure 1. Pre-fermentation wort samples were collected after clarification in the whirlpool. Cooled wort (21°C) was equally divided into four, 13.75 L portions and transferred into 19 L stainless steel fermentation kegs (Kegco; San Diego, CA). Yeast (78 mL) was added to each primary fermenter. PEP was diluted with sterile water (1:5) and then added before fermentation at four different dosages: 0 μL/L (no PEP control), 17 μL/L (half dose PEP), 34 μL/L (full dose PEP, as recommended by the manufacturer) and 68 μL/L (double dose PEP). Beer was fermented at 20°C for 12 days (primary fermentation stage) and then transferred to a new set of 19 L stainless steel fermentation kegs, leaving most of the sediment behind in the primary fermenters. Beer was aged at 4°C for an additional 8.5 weeks (60 days, secondary fermentation stage). Beer samples were syphoned from kegs after 72 days total fermentation and stored immediately in a −20°C freezer located in the pilot plant. At the end of the ageing

stage, a NOVOX 200 plate and frame filter (Vintner's Vault; Paso Robles, CA) with 10 cm × 10 cm plates and 2.0-µm filter pads, containing a mixture of diatomaceous earth and perlite, was used to clarify all beer samples. Physical and chemical properties of the wort and beer can be found in Supplementary Table 1.

Immunochemical analyses

Gluten concentrations in wort and beer samples were measured using the following ELISA kits: Morinaga Wheat/Gluten (Gliadin) (Morinaga Institute of Biological Sciences; Yokohama, Japan), RIDASCREEN® Gliadin (R-Biopharm AG; Washington, MO), and RIDASCREEN® Gliadin competitive (kit properties provided in Supplementary Table 2). ELISA analyses of each sample were conducted in triplicate following the manufacturer's instructions and read in duplicate wells with a ELX808 Ultra Microplate Reader (Bio-Tek Instruments, VT) using wavelengths specified in each kit. Samples were diluted as needed to ensure that the gluten concentrations were within the dynamic range of each kit per the manufacturer's guidelines. Statistical analyses performed on the ELISA results are outlined in the Supplementary Material.

For western blot analysis, beer or wort samples (200 µL) were mixed with an equal volume of Novex Tris-Glycine SDS sample buffer (Invitrogen; Grand Island, NY) and then incubated in a 70°C water bath for 10 min. Each sample (40 µL) was separated under non-reduced conditions by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Novex 4–20% Tris-Glycine gel run at 225 V for 90 min. A SuperSignal™ Enhanced Molecular Weight Protein Ladder was used for molecular weight markers. The proteins were electrotransferred to a 0.45 µm Invitrolon™ PVDF membrane (ThermoFisher Scientific; Waltham, MA) for 90 min at 25V. The membranes were immunoblotted with the detector antibody from the Morinaga ELISA kit diluted 1:10. Chemiluminescence was measured using SuperSignal™ West Dura Extended Duration Substrate, a ChemiDoc™ XRS+ system (Bio-Rad; Hercules, CA), and a 900 sec exposure time.

Mass spectrometry analysis

Mass spectrometric analyses were performed according to a previously described protocol (Panda et al. 2015a; Fiedler et al. 2018). In summary, hydrolytic products and intact proteins were isolated and analysed separately. An aliquot of each sample (100 µL) was passed through a 30 kDa molecular weight cut off (MWCO) filter to collect the products of hydrolysis and enzymatic processing (<30 kDa). Acetone precipitation was used to isolate intact proteins from another 100 µL aliquot of each sample and then the protein pellet was washed with 0.5 M NaCl and water to remove water soluble proteins, such as albumins and globulins, and residual hydrolytic products (Fiedler et al. 2014). Intact proteins were then reduced, alkylated, and digested with chymotrypsin. Hydrolytic products from the MWCO preparation and the chymotryptic peptides from the digested preparation were then separately analysed by LC-MS/MS. Each sample was injected onto a Symmetry C18 M-class trap column (5 µm, 300 Å, 180 µm × 20 mm at 25°C) using a Waters nanoAcquity UPLC and an isocratic gradient at 0.5% B for 3 min at 5 µL/min (solvent A: Optima LC/MS 0.1% formic acid in water, solvent B: Optima LC/MS 0.1% formic acid in acetonitrile). The peptides were then separated on a BEH C18 PicoFRIT column (1.7 µm, 130 Å, 100 µm × 100 mm at 25°C, New Objective; Woburn, MA) using a gradient of 3–40% B over 60 min at 300 nL/min and analysed with a Thermo Q Exactive (QE) mass spectrometer equipped with a Proxeon Nanospray Flex source. A Top10 data-dependent acquisition mode was used with instrument and method parameters as described previously (Fiedler et al. 2018). MS results were searched and filtered using Proteome Discoverer (PD) 2.1 (Thermo; Waltham, MA) as described previously (Fiedler et al. 2018). Briefly, a no-enzyme SEQUEST HT search was performed against a protein database containing all Swiss-Prot and TrEMBL entries from Poaceae, Cannabaceae, and *Saccharomyces cerevisiae* downloaded from

Uniprot (April 2017). The results were filtered to only include peptides associated with gluten proteins (keywords: gliadin, glutenin, hordein, avenin, secalin, prolamin, LMW, and HMW). The two different preparations of each sample, MWCO and digested, were performed and analysed in duplicate and only gluten peptides that were identified by MS in both technical replicates were used. Peptide peak areas were normalised to angiotensin-I which was added to each sample at a concentration of 500 fmol/μL as an injection internal standard to account for run-to-run variation in instrument performance. Native immunopathogenic sequences were downloaded from AllergenOnline CELIAC Database, version 2 (Feb 2018) (Goodman et al. 2016). Fifty-five out of the 465 peptides in the database are designated as barley; however, 111 of the peptides are present in barley proteins (UniProt taxonomy 4512 downloaded Sep 2018). The proline content of full length hordein sequences in the UniProt database was determined to be approximately 15% for γ-hordeins (>30 kDa), 16% for B-hordeins (>28 kDa), 27% for C-hordeins (>30 kDa), and 11% for D-hordeins (>70 kDa).

Results and discussion

Three independent brew trials were conducted to investigate the effects of PEP addition and filtration with diatomaceous earth/perlite on gluten in beer brewed with barley malt. The pilot-scale production process employed is illustrated in Supplementary Figure 1. Barley malt beer was brewed in the absence or presence of 17 (half dose), 34 (full dose), or 68 (double dose) μL Brewers Clarex®/L of wort. The Morinaga, R5 sand, and R5 comp ELISA kits were used to detect gluten in the beer samples (Supplementary Table 3). Even though sandwich-based ELISA methods are not able to accurately quantify the total gluten content, they can still be used to monitor intact gluten proteins. Intact gluten proteins were also characterised by western blot analysis and mass spectrometry. For LC-MS/MS analysis, intact gluten proteins were precipitated with acetone and subjected to a bottom-up proteomic approach via digestion with chymotrypsin. Hydrolysed peptides (<30 kDa) were separately analysed by LC-MS/MS after isolation with a molecular weight cut off filter. The number of gluten peptides that were identified in the two different sample preparations can be found in Supplementary Table 4 and a list of all peptides identified by MS can be found in Supplementary Table 5.

The wort from each brew trial was analysed after the whirlpool stage of the brewing process to characterise the gluten before the addition of PEP, fermentation, and filtration. ELISA results from the Morinaga and R5 sand for brew trials 1–3 are illustrated in Figure 1(a), along with the number of chymotryptic peptides that were produced and identified from intact gluten proteins. ELISA results from the R5 comp are illustrated in Figure 1(b) along with the number of hydrolysed gluten peptides that were identified in the unfermented beer (wort samples obtained after clarification in the whirlpool). These results demonstrate that the gluten composition at the start of fermentation and before PEP addition was slightly different for the three brew trials and was a mixture of intact and hydrolysed gluten, as expected since hordeins are hydrolysed during malting and mashing (Bamforth 2003).

ELISA and MS results before and after filtration with a mixture of diatomaceous earth and perlite (effective pore size of 0.45 μm) for beer brewed in the absence and presence of varying dosages of PEP are illustrated in Figure 2. Beer brewed in the presence of a half dose of PEP was not analysed by mass spectrometry. The LOQ of each ELISA is designated with a dotted line. The Morinaga has the lowest LOQ at 0.27 mg kg^{-1}, the LOQ of the R5 sand is 5 mg kg^{-1}, and the R5 comp has the highest LOQ at 10 mg kg^{-1}. According to both the sandwich and competitive-based ELISAs, diatomaceous earth/perlite filtration significantly reduced the gluten concentration for all beer samples ($p < .05$). The filtration procedure appeared to reduce the gluten concentration in beer brewed in the presence of PEP to a greater extent than for beer brewed in the absence of PEP. The detectable gluten concentration was ≥LOQ of each ELISA method before filtration for all the beer samples, except for the beer brewed in the presence of a double dose of PEP in brew trial 2 as measured by the R5 sand. After filtration, the detectable gluten concentration was reduced in beer brewed in the absence of PEP,

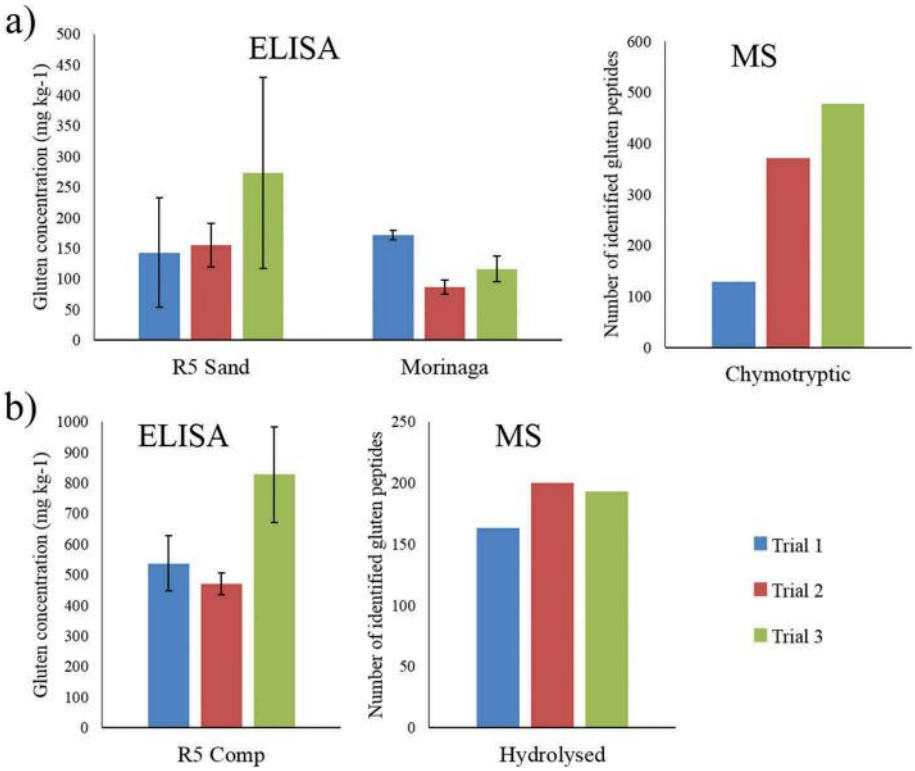

Figure 1. Gluten ELISA and MS results for wort after the whirlpool stage of the brewing process from analyses targeting intact gluten proteins (a) and hydrolysed gluten (b).

but the results remained above the LOQ of each ELISA kit. In contrast, after filtration the detectable gluten concentration fell below the LOQ of the R5 sand for all three brew trials of the PEP-treated beers, and below the LOQ of the Morinaga in brew trial 2. The detectable gluten concentrations in filtered, PEP-treated beer were near or slightly above the LOQ of the Morinaga in brew trials 1 and 3 and the LOQ of the R5 comp in all three brew trials. Differences between the three brew trials demonstrate the hydrolytic variability that can occur during mashing, fermentation, and PEP treatment.

Western blot analysis with the Morinaga antibody (Figure 3) supported the sandwich-based ELISA results and also suggested that the combination of PEP addition and filtration with a mixture of diatomaceous earth and perlite was more effective than each of the treatments alone at reducing the concentration of detectable gluten. Barley gluten is typically comprised of 32% γ-hordeins, 27% B-hordeins (28–35 kDa), 36% C-hordeins (40–50 kDa), and 5% D-hordeins (70–90 kDa) (Koehler et al. 2014). Hordein bands observed for beer brewed in the absence of PEP were similar albeit less intense to the hordein bands observed for the unfermented beer (wort), before filtration. Treatment at the various dosages of PEP produced an intense set of partially hydrolysed hordein bands. A different set of hordein bands at 25–60 kDa were observed in beer brewed in the presence of PEP compared to beer brewed in the absence of PEP, before filtration. After filtration, hordein bands were observed in beer brewed in the absence of PEP, whereas the filtered PEP containing beer were mostly devoid of gluten, except for a faint smear of hordein bands at 30–40 kDa for brew trials 2 and 3 of beer brewed in the presence of a full dose of PEP. This same set of hordein bands was also observed in filtered beer brewed in the presence of a double dose of PEP for brew trial 2. Perlite is known to act like a mass filter and favours the removal of smaller particles versus larger particles as compared to diatomaceous earth, which acts like a sieve and selectively removes larger particles (Freeman 2010). Perhaps the set of smaller, partially hydrolysed intact gluten proteins produced by PEP are more effectively

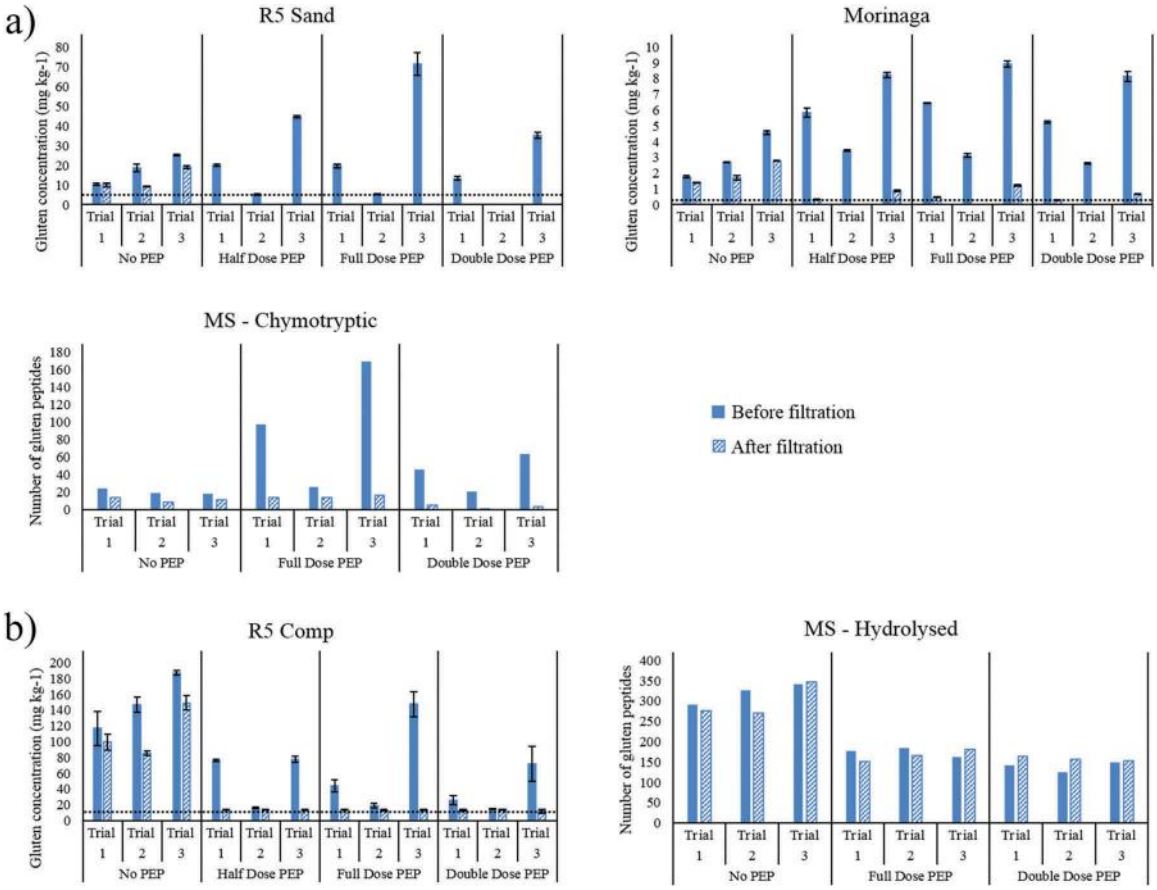

Figure 2. Gluten ELISA and MS results for final beer samples brewed in the presence of various dosages of PEP, before and after diatomaceous earth/perlite filtration, in brew trials 1-3 from analyses targeting intact gluten proteins (a) and hydrolysed gluten (b). The LOQ of each ELISA kit is designated with a dotted line.

removed by perlite compared to the larger intact gluten proteins that are present in untreated beer.

MS results for the number of chymotryptic peptides generated from intact or partially hydrolysed gluten proteins trend closely with results from the sandwich-based ELISAs. In contrast, a similar number of hydrolysed gluten peptides were identified before and after filtration for all the beer samples.

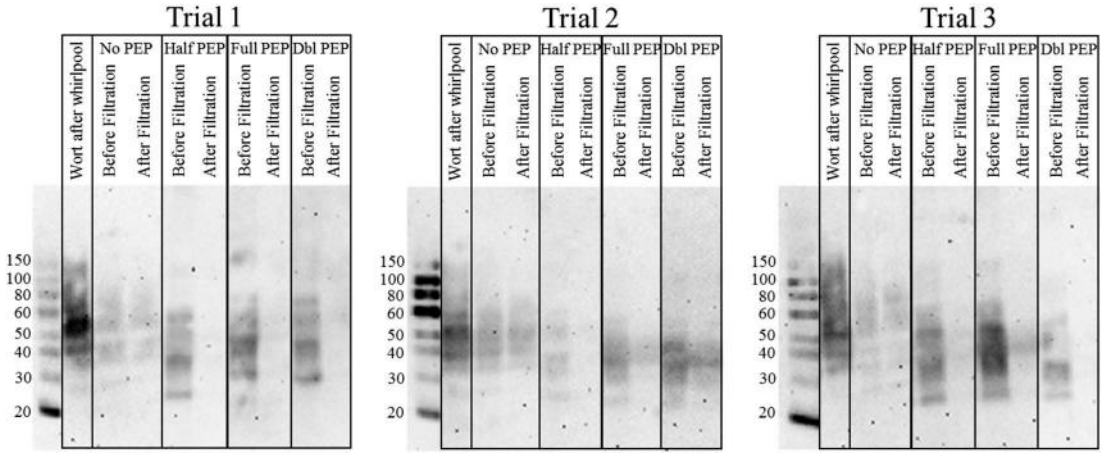

Figure 3. Western blot analysis of wort samples after the whirlpool stage and final beer samples before and after filtration for beer brewed in the absence and presence of various dosages of PEP.

Normalised peak areas of individual hydrolysed peptides were tracked to determine the effect of filtration. The MS method is unable to determine the absolute gluten concentration, but it can be used for the relative quantification of particular peptides between samples. Supplementary Figure 2 illustrates the average percent of peak area remaining after filtration compared to the peak area before filtration for gluten peptides that were identified in each beer before and after filtration in all three brew trials. The trends observed for the peptide peak areas were similar to the trends observed for the overall number of hydrolysed gluten peptides in Figure 2. Therefore, not all forms of hydrolysed gluten were removed by filtration. The results agree with literature where hydrolysed gluten peptides have been detected in commercial PEP-treated beers that were likely filtered before bottling (Colgrave et al. 2017).

The enzymatic action of PEP was confirmed by analysis of the hydrolysed gluten peptides identified by mass spectrometry after filtration. The number of hydrolysed gluten peptides that terminate in a proline residue increased from an average of 20 ± 1% in beer brewed in the absence of PEP across the three brew trials to 42 ± 4% and 43 ± 1% in beer brewed in the presence of a full dose and double dose of PEP, respectively (Supplementary Figure 3). Additionally, fewer internal proline residues (P-X motifs) were observed in the hydrolysed gluten peptides in beer brewed in the presence of PEP versus beer brewed in the absence of PEP (Supplementary Figure 4). The number of hydrolysed gluten peptides that contained one or more internal proline residues decreased from an average of 89 ± 2% in beer brewed in the absence of PEP across the three brew trials to 74 ± 4% and 69 ± 1% in beer brewed in the presence of a full dose and double dose of PEP, respectively. However, internal proline residues represent missed cleavage sites for PEP and demonstrate that PEP digestion was incomplete in the PEP containing beer.

Figure 4 illustrates the hydrolysed gluten peptides identified by MS in the final beer sample after filtration according to their assigned hordein class. More hydrolysed gluten peptides assigned to D-hordeins were identified in the filtered, PEP containing beer compared to hydrolysed gluten peptides assigned to γ-, B-, and C-hordeins. This could be explained by the proline contents of the different hordein classes. According to literature, γ-hordeins are approximately 17% proline, B-hordeins are 19% proline, C-hordeins are 29% proline, and D-hordeins are 10% proline (Koehler et al. 2014). A complete *in silico* digestion of the hordein sequences in Uniprot was performed with PEP to determine how many peptides would be produced that are greater than 9 amino acids in length, the required length for a T-cell epitope (Sollid et al. 2012). C-hordeins would only produce an average of 3 peptides ≥9 amino acids in length, while γ- and B-hordeins would produce an

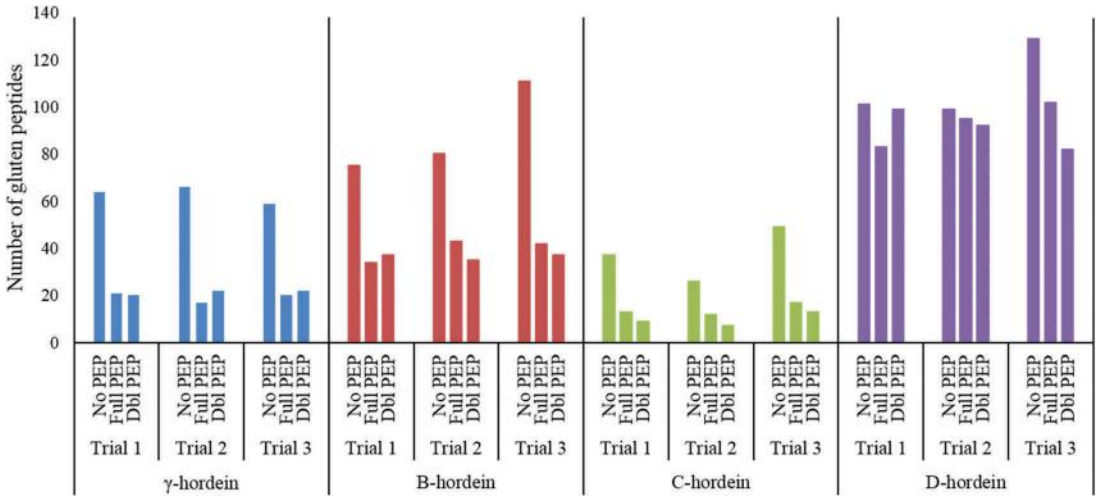

Figure 4. Hydrolysed gluten peptides identified by MS in the final beer samples after filtration according to their assigned hordein class.

average of 8 and 9 peptides, respectively. D-hordeins, on the other hand, would produce an average of 37 peptides of sufficient size to elicit an autoimmune response upon digestion with PEP.

The hydrolysed and chymotryptic gluten peptides identified by MS were searched against a database of native gluten peptides from wheat, barley, rye, and oats that are associated with CD using a custom Python script (Goodman et al. 2016). Table 1 shows the total number of gluten peptides identified in each beer sample, the number of gluten peptides that contained a ≥9 amino acid (aa) match to a known immunopathogenic sequence, and the number of gluten peptides that contained a full match to a known immunopathogenic sequence. A list of the potentially immunopathogenic gluten peptides from Table 1 can be found in Supplementary Table 6. Peptides that contained a ≥9 aa match to a known immunopathogenic sequence were considered because nine amino acids is the minimum length of a T-cell epitope (Sollid et al. 2012). Fewer hydrolysed gluten peptides that contained immunopathogenic sequences were observed in the PEP containing beer compared to the non-PEP containing beer. However, hydrolysed gluten peptides that contained ≥9 aa matches to known immunopathogenic sequences were found in all PEP containing beer even after filtration. One hydrolysed C-hordein peptide that matched a 15-aa stretch of a known hordein immunopathogenic sequence was found in all three brew trials of the filtered beer brewed in the presence of the full dose of PEP (Figure 5(a)). Hydrolysed D-hordein peptides were also identified in the PEP containing beers that contained homologous regions to known immunopathogenic sequences, but sequence differences between barley and wheat gluten proteins prevented direct matches. Figure 5(b) shows a hydrolysed D-hordein peptide aligned to two known immunopathogenic sequence from HMW-glutenin. A number of sequence variants of the wheat derived epitope have been shown to stimulate T-cells isolated from a CD patient (van de Wal et al. 1999), but it is not known how the immunopathogenicity of the epitope is affected by insertions. Figure 5(c) shows two hydrolysed D-hordein peptides identified in brew trial 1 of the filtered beer brewed in the presence of a double dose of PEP. In this instance, PEP cleavage was observed in the central portion of the immunopathogenic sequence, which probably inactivates the epitope according to a truncation analysis performed by van de Wal et al. on the HMW-glutenin epitope (van de Wal et al. 1999). Supplementary Figure 5 illustrates a comparison of all the hydrolysed gluten peptides identified in the three different brew trials of the filtered beer

Table 1. Gluten peptides identified by MS that contained known immunopathogenic sequences.

			Before Filtration			After Filtration		
				Contained immunopathogenic seq.			Contained immunopathogenic seq.	
	PEP Dosage	Brew Trial	Total no. gluten peptides	≥9 aa match	Full match	Total no. gluten peptides	≥9 aa match	Full match
Hydrolysed	No PEP	1	291	23	28	277	24	29
		2	326	27	29	271	25	23
		3	341	33	31	348	38	26
	Full PEP	1	178	10	–	151	13	–
		2	185	12	1	167	11	–
		3	163	11	–	181	14	–
	Double PEP	1	142	8	–	165	13	–
		2	125	10	–	156	12	–
		3	150	14	–	154	10	–
Chymotryptic	No PEP	1	24	–	–	14	–	–
		2	19	–	–	9	–	–
		3	18	–	–	11	1	–
	Full PEP	1	97	1	3	14	1	–
		2	26	1	–	14	1	–
		3	169	4	6	17	2	–
	Double PEP	1	46	1	1	5	1	–
		2	21	1	–	2	–	–
		3	63	1	1	4	1	–

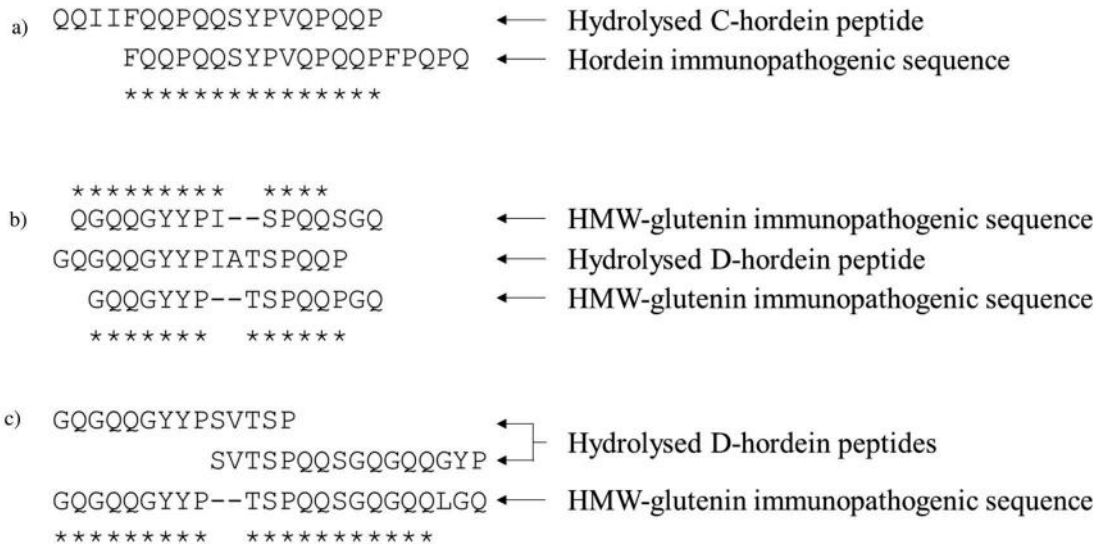

Figure 5. Hydrolysed gluten peptides identified in filtered PEP containing beer aligned with known immunopathogenic sequences.

brewed in the presence of a full dose of PEP as well as the immunopathogenic sequence containing hydrolysed gluten peptides. Overall, these results indicate that the gluten peptide profiles varied between the replicate brew trials demonstrating the hydrolytic variability between each of the brewing trials.

Figure 6 illustrates the sequence coverage observed for a B3-hordein in the digested preparation of beer brewed with a full dose of PEP before filtration for each of the three brew trials. A 14-aa stretch that matched a known immunopathogenic sequence from LMW-glutenin was observed in brew trial 3 that spanned two chymotryptic gluten peptides. Chymotrypsin, which preferentially cleaves C-terminally to F, W, and Y (except when followed by proline with low affinity for L, M, and H), cleaved in the middle of the immunopathogenic sequence. The chymotryptic peptides that were detected by MS were generated from intact or partially hydrolysed hordeins during the sample preparation; therefore, the intact immunopathogenic sequence was most likely present in the beer. Sequence differences between wheat and barley gluten proteins prevented a longer match to the immunopathogenic sequence. The LQQQCSPVAM(SQ) peptide from B-hordein was the only chymotryptic peptide observed after filtration, which suggests that some of the low molecular weight gluten proteins were not completely removed by the final filtration step.

Conclusion

The reliable and accurate detection and quantification of gluten in hydrolysed and fermented foods is challenging using available ELISA technology alone. Therefore, alternate methods, such as mass spectrometry, are needed for a more complete examination of

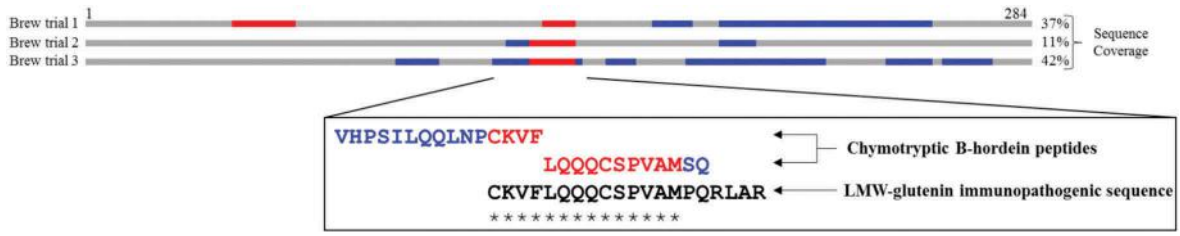

Figure 6. Sequence coverage observed for a B3-hordein (I6SW30) in the digested preparation of beer brewed with a full dose of PEP before filtration. Chymotryptic peptides that were detected in each brew trial are highlighted in blue and sequences that were identified that match known immunopathogenic sequences are highlighted in red.

gluten in beer. The combination of PEP addition and filtration with a mixture of diatomaceous earth seemed to provide the greatest reduction in the amount of detectable gluten. Still, intact gluten proteins were detected in filtered, PEP-treated beer and the presence of a subset of hydrolysed gluten peptides identified by MS was not affected by the diatomaceous earth/perlite filtration. Gluten peptides containing potentially immunopathogenic sequences were identified in the PEP containing beers, even after the used filtration technique. However, detection of gluten peptides that contain immunopathogenic sequences does not guarantee that they will elicit a reaction in individuals with CD. The immunopathogenicity of the entire identified gluten peptide needs to be confirmed because flanking residues can affect epitope binding and the homologous region may not contain the core binding motif. *In vitro* studies and perhaps clinical studies in humans are needed to determine if the gluten present in PEP containing beer can elicit a reaction in individuals with CD. Hydrolytic variability was observed between the three brew trials in this study, which suggests that the gluten profile may change from batch to batch depending on the various processes involved in beer production. These results further highlight the need for complementary analytical approaches to ensure that gluten and gluten peptides with potential immunopathogenic sequences are removed during the manufacture of gluten-reduced beer.

Acknowledgments

Appreciation is expressed for the technical support provided by UW-Madison for their work in producing the pilot plant beer used in this study. The authors also acknowledge the assistance provided by Joe Casey (Craft Brewers Alliance) and Sylvie van Zandycke (Lallemand) for input in the conditions used in the brewing trials. The authors would also like to thank James Pettengill for writing custom Python scripts that were used to analyse the MS results. This project was supported by the U.S. Food and Drug Administration (FDA) and the Institute for Food Safety and Health (IFSH). This project was also supported in part by an appointment to the Research Participation Program at CFSAN administered by ORISE through an interagency agreement between the U.S. DOE and the U.S. FDA.

Disclosure Statement

No potential conflict of interest was reported by the authors.

Notes

This article is not an official U.S. Food and Drug Administration (FDA) guidance or policy statement. No official support or endorsement by the U.S. FDA is intended or should be inferred.

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