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Liquid Chromatography–Mass Spectrometry Analysis Reveals Hydrolyzed Gluten in Beers Crafted To Remove Gluten

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Supporting Information

ABSTRACT: During brewing, gluten proteins may be solubilized, modified, complexed, hydrolyzed, and/or precipitate. Gluten fragments that persist in conventional beers render them unsuitable for people with celiac disease (CD) or gluten intolerance. Barley-based beers crafted to remove gluten using proprietary precipitation and/or application of enzymes, e.g. prolyl endopeptidases (PEP) that degrade the proline-rich gluten molecules, are available commercially. Gluten measurement in fermented products remains controversial. The industry standard, a competitive ELISA, may indicate gluten values <20 mg/kg, which is deemed safe for people with CD. However, in this study, liquid chromatography–mass spectrometry analyses revealed gluten peptides derived from hydrolyzed fragments, many >30 kDa in size. Barley gluten (hordeins) were detected in all beers analyzed with peptides representing all hordein classes detected in conventional beers but also, alarmingly, in many gluten-reduced beers. It is evident that PEP digestion was incomplete in several commercial beers, and peptides comprising missed cleavages were identified, warranting further optimization of PEP application in an industrial setting.

KEYWORDS: *gluten, prolyl endopeptidase (PEP), beer, liquid chromatography–mass spectrometry (LC–MS)*

INTRODUCTION

Celiac disease (CD) is an inflammatory disorder of the small intestine affecting 1% of people in Western populations.[1] After exposure to gluten via ingestion, an inappropriate immune response results in destruction of the microvilli within the intestine. This leads to conditions commonly involving malabsorption of nutrients (anemia, osteoporosis), gastrointestinal complaints (diarrhea, bloating) and skin conditions (dermatitis), through to endocrine, neurological, and reproductive disorders.[2] The only treatment for people with CD is a strict gluten-free (GF) diet.

Brewing is considered the oldest biotechnological process known to mankind. Beer represents the third most popular beverage after water and tea. The sugars released from malted barley serve as the primary nutrient source for yeast during fermentation when they are converted into alcohol. Proteins, predominantly from barley and to a lesser extent yeast, that persist in beer have important contributions toward end product quality, including haze formation, foam retention, foam stability, and flavor. The dominant proteins identified include the serpins, lipid transfer proteins (LTPs), α-amylase/trypsin inhibitors, and storage proteins, including gluten.[3−7] In the context of beer, gluten is the name for the storage proteins found in barley (hordein), wheat (gliadin/glutenin), and rye (secalin). Strict gluten avoidance in CD precludes the consumption of beers made from barley, wheat, and/or rye. There are a number of beers made from nongluten-containing cereals (corn, rice, sorghum, millet) or pseudocereals (buckwheat); however, these products often lack the distinctive flavor and aroma imparted by malted barley.

It is well-established that proteins undergo a number of modifications and hydrolysis during the brewing process, especially during malting and mashing.[8,9] A large proportion of the protein content is removed from wort during boiling and during wort cooling.[10,11] The hordeins are reduced by >30% during malting (up to 65% for the C-hordeins)[12] and further during brewing.[13] In a controlled study, gluten content was shown to decrease by 46−79% from first wort to beer.[14] The modification of gluten during brewing has been comprehensively reviewed by Kerpes et al.[15] Extensive hydrolysis, however, does not abolish the epitopes that are known to trigger CD, and several studies have reported on Celiac responses to commercial beers.[5,16−18]

A range of brewing aids that are used to stabilize beers, through disrupting the polyphenol–protein interactions that lead to haze formation, have also seen application in gluten removal. These include the use of polyvinylpolypyrrolidone (PVPP) and silica gel or condensed tannins (proanthocyanidins), and their use has led to a reduction in the gluten content of treated beers.[10,11,19] The enzyme transglutaminase (TG) has been employed as a means of detoxifying food and beverages.[20,21] For example, microbial TG creates cross-links between gluten proteins/peptides that ultimately results in the precipitation of these proteins, allowing their removal by filtration.[22]

Researchers have applied endogenous peptidases from germinated wheat, rye, and barley, demonstrating cleavage of celiac-active epitopes.[23,24] In recent years, it has become common practice to generate gluten-reduced or gluten-free barley-based beers through the addition of enzymes during the brewing process, commonly added at the start of fermentation.

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A commercial preparation of a prolyl endopeptidase (PEP, also known as prolyl oligoprotease) from *Aspergillus niger* (referred to as AN-PEP) was first used to debitter protein hydrolysates[25] and subsequently to decrease haze by hydrolyzing haze-sensitive proteins. PEPs including AN-PEP cleave proteins at proline (Pro, P) residues[25] and are able to degrade gluten owing to the high frequency of Pro (10−30%) found in gluten proteins.[26,27] The enzymatic detoxification of gluten has been recently reviewed by Wieser et al.[28]

In a series of studies employing the R5 competitive ELISA,[24,29,30] a range of commercial beers was analyzed, including those produced from nongluten-containing grains and from barley with and without PEP treatment. Of these, the beers employing PEP treatment were shown to yield a gluten content below the CODEX threshold of 20 mg/kg. A further study examining the action of AN-PEP on the degradation of gluten peptides[31] employed ELISA for gluten quantitation and LC−MS to follow the fate of the gluten peptides qualitatively. Analysis of untreated and AN-PEP-treated beers revealed that immunotoxic epitopes were present in the untreated beers but not in the AN-PEP-treated beers. Another study examined the effectiveness of AN-PEP by both ELISA and LC−MS in a sorghum beer incurred with wheat gluten.[32] In control beers, a gradual reduction (fourfold) in gluten content was demonstrated over the first three days of fermentation, whereas AN-PEP-treated beers showed a marked decrease (>15-fold) in gluten content from 3 to 14 days. Using Western blotting, the HMW-glutenins were shown to be less susceptible to AN-PEP than the LMW-glutenins. From these studies and similar applications in wheat, bran, and foodstuffs,[33,34] it is apparent that PEP is able to degrade gluten; however, it is unclear if all potential immunopathogenic sequences are completely eliminated. Moreover, the safety of gluten-reduced beers is still contentious, in part owing to questions regarding the accuracy of testing fermented and hydrolyzed foods and the ability to equate hydrolyzed gluten content to an equivalent amount of intact gluten. This latter issue is addressed by the United States FDA proposed rule (FDA-2014-N-1021).[35] A recent study on the antibody response to gluten-reduced beers found that serum from active-CD patients bound to residual gluten peptides in conventional beers and that a subset of the patient sera also reacted to gluten-removed beers.[18]

In the current study, LC−MS analysis was applied to a selection of gluten-reduced and gluten-free commercial barley-based beers to determine the effect of gluten reduction treatments on the protein and peptide profiles.

MATERIALS AND METHODS

Reagents and Test Samples. Chemicals, including formic acid (FA), ammonium bicarbonate, dithiothreitol, and iodoacetamide, were purchased from Sigma-Aldrich (Sydney, NSW, Australia). Acetonitrile was purchased from ChemSupply (Gillman, SA, Australia). Enzymes used for digestion (trypsin and chymotrypsin) were purchased from Promega (Sydney, NSW, Australia). A selection of beers was purchased internationally from commercial liquor stores based on their ingredients and gluten status according to their packaging and/or company Web site. All beers selected were barley-malt based products rather than gluten-free beers based on nongluten containing grains such as rice, sorghum, millet, or tef. A number of regular beers that had previously[6] been shown to contain gluten were selected as positive controls, C1−C4. The gluten-reduced (or low gluten, LG) beers, LG1-LG7 and LG9-LG11, were PEP-treated. LG8 is manufactured by an undisclosed proprietary process. LG12 is brewed with a novel ultralow gluten barley.[36]

Digestion of Whole Beers. Whole beers (n = 4 technical replicates) were subjected to enzymatic digest using either trypsin or chymotrypsin. Aliquots of degassed beer (50 μL) were diluted 1:1 in 50 mM ammonium bicarbonate containing 1 mM $CaCl_2$, pH 8.5 (50 μL). To these solutions, 10 μL of 50 mM dithiothreitol was added, and the samples were incubated at 60 °C for 30 min. Subsequently, the samples were cooled; 10 μL of 100 mM iodoacetamide was added, and the samples were incubated at RT for 20 min in the dark. To these solutions, 10 μL of either trypsin or chymotrypsin (1 μg/μL) was added with incubation at 37 °C for 16 h. To quench the digestion, 50 μL of 1% formic acid was added, and the samples were stored at −20 °C until analysis. A <10 kDa fraction was generated by passing 50 μL aliquots (n = 4) of the reduced, alkylated beer (prior to digestion) through 10 kDa molecular weight cutoff (MWCO) filters (Millipore, Sydney, Australia) by centrifugation at 20 800g for 15 min. The filtrates were taken and processed as described above with the exception that instead of enzyme, 10 μL of ammonium bicarbonate was added.

Protein Size Fractionation and Digestion. The beers to be assessed (n = 4 replicates) were subjected to size fractionation. Each beer (200 μL) was diluted 1:1 in 50 mM ammonium bicarbonate. To the beers, 10 μL of 410 mM dithiothreitol (final concentration 10 mM) was added with vortex mixing and incubation at 60 °C for 30 min. The cysteines were alkylated using 10 μL of 1050 mM iodoacetamide (final concentration 25 mM) with vortex mixing and incubation at room temperature in the dark for 20 min. From each reduced and alkylated beer sample, 100 μL aliquots were applied to 30 kDa MWCO filters by centrifugation at 20 800g for 10 min. The filters were then washed with 100 μL of 50 mM ammonium bicarbonate, pH 8.5 with centrifugation at 20 800g for a further 10 min. The filtrates containing proteins <30 kDa were transferred to 10 kDa filters and centrifuged and washed as described above. The filtrates containing proteins/peptides <10 kDa were transferred to clean tubes, and 10 μL of 1 μg/μL trypsin was added with incubation at 37 °C for 16 h. The protein fraction remaining on the 10 kDa (∼10−30 kDa) and 30 kDa (>30 kDa) MWCO filters were digested by addition of 200 μL of 50 μg/mL trypsin in 50 mM ammonium bicarbonate with incubation at 37 °C for 16 h. The digested peptides were collected by centrifugation at 20 800g for 10 min. The filters were then washed with 200 μL of 50 mM ammonium bicarbonate, pH 8.5 with centrifugation at 20 800g for a further 10 min. All samples were lyophilized and stored at −20 °C until analysis.

LC−MS/MS Analysis for Protein Identification. The digested samples were reconstituted in 100 μL of 1% formic acid, and aliquots (5 μL) of each replicate were pooled for data-dependent analysis. From these, an aliquot (5 μL) was chromatographically separated on an Ekspert nanoLC415 (Eksigent, Dublin, CA, United States) directly coupled to a TripleTOF 6600 MS (SCIEX, Redwood City, CA, United States). The peptides were desalted for 5 min on a ChromXP C18 (3 μm, 120 Å, 10 × 0.3 mm) trap column at a flow rate of 10 μL/min solvent A and separated on a ChromXP C18 (3 μm, 120 Å, 150 mm × 0.3 mm) column at a flow rate of 5 μL/min. The solvents used were (A) 5% DMSO, 0.1% formic acid, 94.9% water and (B) 5% DMSO, 0.1% formic acid, 90% acetonitrile, 4.9% water. A linear gradient from 5 to 45% solvent B over 40 min was employed followed by 45−90% B over 5 min, a 5 min hold at 90% B, return to 5% B over 1 min, and 14 min of re-equilibration. The eluent from the HPLC was directly coupled to the DuoSpray source of the TripleTOF 6600 MS. The ion spray voltage was set to 5500 V; the curtain gas was set to 138 kPa (20 psi), and the ion source gas 1 and 2 (GS1 and GS2) were set to 103 and 138 kPa (15 and 20 psi). The heated interface was set to 100 °C. Data were acquired in information-dependent acquisition (IDA) mode. The IDA method consisted of a high-resolution time-of-flight (TOF)-MS survey scan followed by 30 MS/MS scans, each with an accumulation time of 40 ms. First stage MS analysis was performed in positive ion mode over the mass range of m/z 350−1800 with a 0.25 s accumulation time. Tandem mass spectra were acquired on precursor ions that exceeded 200 counts/s with charge state 2−5. Spectra were acquired over the mass range of m/z 100−2000 using the manufacturer's rolling collision energy (CE) based on the size and

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charge of the precursor ion and a collision energy spread (CES) of 5 V for optimum peptide fragmentation. Dynamic ion exclusion was set to exclude precursor ions after one occurrence with an 8 s interval and a mass tolerance of 50 ppm, and peaks within 6 Da of the precursor mass were excluded.

Protein Identification. Protein identification was undertaken using a thorough search effort (which considers all Unimod modifications) using ProteinPilot 5.0 software (SCIEX) with the Paragon algorithm.[37] Depending on the sample processing, iodoacetamide or none was selected as the alkylating agent, and trypsin, chymotrypsin, or no enzyme was selected as the digestion enzyme. Tandem mass spectrometry data were searched against a database comprising Uniprot-Poaceae proteins (version 2017/02) appended with custom gluten database[6] (2 891 190 sequences). The database search results were manually curated to yield the protein identifications using a 1% global false discovery rate (FDR) determined by the in-built FDR tool within ProteinPilot software.[38]

LC−MS/MS Analysis for Relative Protein Quantitation. The individual replicates (whole beers: 10 μL or size-fractionated beers: 5 μL) were chromatographically separated on an UHPLC system (Shimadzu Nexera, Sydney, Australia) directly coupled to a QTRAP 6500 mass spectrometer (AB SCIEX, Foster City, United States). The samples were analyzed by scheduled multiple reaction monitoring (MRM) using methods previously described.[6,39] The cycle time was set to 0.3 s, and the MRM transitions were scheduled to be monitored within 60 s of their expected retention time (RT, ±30 s). Peaks were integrated using MultiQuant software v3.0 (SCIEX). Peptide peak area variability is expressed as a coefficient of variation (CV) and is calculated based on the summed XIC areas of three MRM transitions for each peptide across the technical replicates (n = 4). The peak areas were exported to Microsoft Excel, and the peak area was summed for the three transitions per peptide. Graphical images were generated in Graphpad Prism using the mean (±SD) summed peak area.

RESULTS AND DISCUSSION

Identification of Gluten in Beers. The major proteins detected in the tryptic digests of whole beer were lipid transfer proteins (LTPs), storage proteins (globulins), serpins, and a suite of α-amylase/trypsin inhibitors, as is typical in the analysis of beers.[6] For each beer, a range of peptides derived from gluten was detected and identified (Supplementary Table 1). Some of these peptides were fully tryptic, i.e. cleaved at both ends by trypsin, indicating that they had been cleaved from larger polypeptides and/or proteins. Other peptides were semitryptic, i.e. cleaved at only one end by trypsin, indicating that they were derived from hydrolyzed gluten present in beer. In the control beers, a range of peptides derived from B-hordeins (examples: I6SJ22; P06470; Q4G3S1), C-hordeins (Q41210; Q40053), D-hordeins (I6TRS8), γ-hordeins (I6TMV6, I6TEV2), and avenin-like proteins (ALP: F2EGD5, M0VEH1) were detected (Supplementary Table 1A). Many of the same proteins (examples: B-hordeins I6SJ22, P06470; C-hordeins Q40053, Q40037; D-hordein I6TRS8; γ3-hordein I6TEV2; ALP M0VEH1) were detected in the gluten-reduced beers. Peptides spanning the entire length of the single gene product D-hordein (I6TRS8) were detected, implying that either the full-length protein persisted in the beers or that multiple protein fragments that harbored the detected peptides were present. To examine the potential size of the protein fragments, size fractionation of the beers was undertaken followed by repeat tryptic digestion and LC−MS/MS analysis. Examining D-hordein (expected MW 75.0 kDa) as detected in a beer crafted to remove gluten (beer LG7), peptides spanning the entire protein length were detected in both the 10−30 kDa and >30 kDa fractions. The results were qualitatively similar (LG7, 40.0% sequence coverage) to that observed for a control barley beer (C1, 37.9% sequence coverage) that underwent no gluten-reduction processes (Figure 1).

(A) Control beer, C1:

MAKRLVLFVAVIVALVALTTAEREINGNNIFLDSRSRQLQCERELQESSLEACRRVVDQQLVGQLPWS
TGLQMQCCQQLRDVSPECRPVALSQVVRQYEQQTEVPSKGGSFYPGGTAPPLQQGGWWGTSVKWYYPD
QTSSQQSWQGQQGYHQSVTSSQQPGQGQQGSYPGSTFPQQPGQGQQPGQRQPWSYPSATFPQQPGQGQ
GQQGYYPGATSLLQPGQGQQGPYQSATSPQQPGQGQGQQEPYPIATSPHQPGQWQQPGQGQQGYYPSV
TSPQQSGQGQQGYPSTTSPQQSGQGQQLGQGQQPGQGQQGYPSATFPQQPGQWQQGSYPSTTSPQQSG
QGQQGYNPSGTSTQQPGQVQQLGQGQQGYYPIATSPQQPGQGQQLGQGQQPGHGQQLVQGQQQGQGQQ
GHYPSMTSPHQTGQGQKGYYPSAISPQQSGQGQQGYQPSGASSQGSVQGACQHSTSSPQQQAQGCQAS
SPKQGLGSLYYPSGAYTQQKPGQGYNPGGTSPLHQQGGGFGGGLTTEQPQGGKQPFHCQQTTVSPHQG
QQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHPGQQTTVSPH
QGQQTTVSPHPGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVS
PHQGQQPGEQPCGFPGQQTTVSLHHGQQSNELYYGSPYHVSVEQPSASLKVAKAQQLAAQLPAMCRLE
GGGGLLASQ

(B) Gluten-reduced beer, LG7:

MAKRLVLFVAVIVALVALTTAEREINGNNIFLDSRSRQLQCERELQESSLEACRRVVDQQLVGQLPWS
TGLQMQCCQQLRDVSPECRPVALSQVVRQYEQQTEVPSKGGSFYPGGTAPPLQQGGWWGTSVKWYYPD
QTSSQQSWQGQQGYHQSVTSSQQPGQGQQGSYPGSTFPQQPGQGQQPGQRQPWSYPSATFPQQPGQGQ
GQQGYYPGATSLLQPGQGQQGPYQSATSPQQPGQGQQEPYPIATSPHQPGQWQQPGQGQQGYYPSV
TSPQQSGQGQQGYPSTTSPQQSGQGQQLGQGQQPGQGQQGYPSATFPQQPGQWQQGSYPSTTSPQQSG
QGQQGYNPSGTSTQQPGQVQQLGQGQQGYYPIATSPQQPGQGQQQLGQGQQPGHGQQLVQGQQQGQGQQ
GHYPSMTSPHQTGQGQKGYYPSAISPQQSGQGQQGYQPSGASSQGSVQGACQHSTSSPQQQAQGCQAS
SPKQGLGSLYYPSGAYTQQKPGQGYNPGGTSPLHQQGGGFGGGLTTEQPQGGKQPFHCQQTTVSPHQG
QQTTVSPHQGQQTTVSPHQGQQTTVSPHPGQQTTVSPHQGQQTTVSPHQGQQTTVSPHPGQQTTVSPH
QGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQPGEQPCGFPGQQTTVSLHHGQQSN
ELYYGSPYHVSVEQPSASLKVAKAQQLAAQLPAMCRLEGGGGLLASQ

Figure 1. Protein sequence coverage of the D-hordein (I6TRS8) as detected in the >30 kDa size fraction of the control beer C1 (A) and the gluten-reduced beer LG7 (B), revealing peptide identifications (bold, underlined >95% confidence) spanning the length of the protein.

The results of the chymotryptic digest revealed a similar suite of protein identifications as obtained after trypsin digestion. There was only a single low gluten beer (LG11) wherein no gluten was detected after chymotryptic digest and a second beer (LG12) wherein only a single peptide from γ3-hordein (I6TEV2) was detected. The most commonly detected gluten proteins were D-hordein (I6TRS8) and γ3-hordein (I6TEV2) in the LG beers and additionally two C-hordeins (Q40053; Q41210) in the control beers (Supplementary Table 1B). We also looked for the presence of epitopes known to bind to the Mendez R5 antibody[40,41] and detected QQPFP, LQPFP, QQPYP, and PQPFP within the identified peptide sequences. Akin to the study of Akeroyd et al.,[31] no peptides containing these antigenic sites were detected in the chymotryptic digest of the gluten-reduced beers, but 58 peptides were detected in the control beers (Supplementary Table 1B).

Table 1 shows the number of gluten-derived peptides identified with ≥95% confidence in the suite of beers tested. As depicted in Figure 2, the control beers show the highest number of unique gluten-derived peptides (range: 54−86 for trypsin; 59−121 for chymotrypsin) with the low gluten beers LG11 and LG12 revealing the least. The spectral count (total number of gluten peptide spectra acquired) followed the same trend as the number of unique peptides but also reflected the abundance of these peptides because the more abundant a peptide is in a sample, the greater the spectral redundancy. Qualitatively, from the low gluten beers tested, LG7 and LG8 were noted to contain the greatest diversity and abundance of gluten-derived peptides, both with approximately twice the number and spectral count compared to the average of all low-gluten beers. LG3−LG6 also revealed values above the average.

The undigested filtrates (<10 kDa) were also analyzed, revealing a range of internal gluten peptide fragments. As no digestion was employed, the termini of the peptides detected may reflect the processes during the brewing of the beers. It is also expected that hydrolyzed gluten will be present in beer, as

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Table 1. Numbers of Confidently Identified Gluten Peptides in Control (C) and Low Gluten (LG) Beers after Enzymatic Digestion

	trypsin (whole beer)		chymotrypsin (whole beer)	
ID	gluten peptides[a]	spectral count[b]	gluten peptides[a]	spectral count[b]
C1	61	221	71	265
C2	69	294	105	438
C3	86	356	121	509
C4	54	204	59	162
LG1	22	100	19	47
LG2	24	85	20	59
LG3	57	189	45	127
LG4	32	124	38	98
LG5	55	196	36	89
LG6	54	177	42	119
LG7	83	357	53	140
LG8	62	356	27	124
LG9	30	81	8	21
LG10	30	79	12	29
LG11	23	47	0	0
LG12	8	20	1	1

[a]Number of unique gluten peptide sequences identified with >95% confidence. [b]Spectral count (number of times spectra acquired) of gluten-derived peptides.

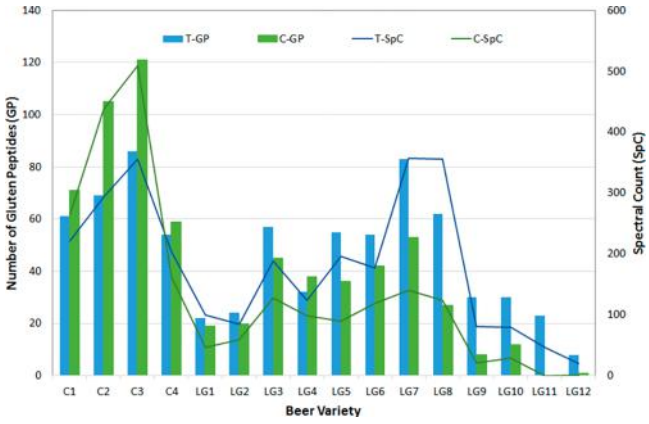

Figure 2. Number of unique gluten peptides (GP) and spectral count (SpC) after trypsin (T, blue) digestion and chymotrypsin (C, green) digestion, as presented in Table 1. Four control (C) and 12 low gluten (LG) whole beers were assessed.

has been observed in previous analyses.[6] Many of the peptides identified in the sub-10 kDa fraction of PEP treated beers were the result of the action of a prolyl endopeptidase with cleavages occurring at P−X (and to a lesser extent X−P), but this was not an efficient process as there were many missed cleavages, that is, peptides present that contained X−P−X motifs (Table 2). The control beers (C1−C4) showed no obvious pattern of protein cleavage with values ranging from 7 to 15% of gluten peptide fragments resulting from hydrolysis of P−X or X−P bonds (Table 2). Moreover, >90% of the gluten peptides detected in C1−C4 contained P−X sites within their sequences. Examining LG1-LG7 and LG9-LG11, the majority of peptide fragments resulted from cleavage at P−X (55−73%) and to a lesser extent X−P (2−9%), indicating the use of PEP in the production of these beers. Reviewing the identified sequences within the PEP treated beer revealed that 60−94% of the gluten peptides contained additional PEP cleavage sites. In fact, only LG11 contained more completely digested peptides than partially digested (42.1% missed cleavages). The other notable fact was that untreated beers contained a higher number of P−X sites within an individual peptide (up to five missed cleavages) compared to PEP treated beers where peptides typically contained only one or two missed cleavage sites. The exception was LG7, which also contained a peptide with five missed cleavages: QQAE-LIIP$^{\downarrow}$QQP$^{\downarrow}$QQP$^{\downarrow}$FP$^{\downarrow}$LQP$^{\downarrow}$HQP. Notably, this peptide also contained the QQPFP epitope recognized by the Mendez R5 antibody. For LG8, PEP activity was not apparent with only 7% of gluten-derived fragments cleaved at P−X or X−P, which is within the same range as the control beers. LG8 also yielded a high proportion of gluten peptide identifications containing P−X sites (82.8%). As with all the beers in this study, LG8 was brewed using barley, but the gluten is claimed to be removed by a proprietary process. Alongside a handful of B- and γ-hordeins, D-hordein was identified confidently in LG8 by 11 peptides that all clustered in the C-terminal region of the protein, suggesting that a C-terminal fragment persists after brewing. In LG12, which is a gluten-free beer brewed using a novel gluten-free barley,[36] only γ3-hordein (I6TEV2) was detected by seven peptide fragments resulting from nonspecific cleavage (hydrolysis during brewing). This was expected because only γ3-hordein is detectable in grain of this novel barley,[35] and only peptides derived from I6TEV2 were identified in the trypsin (8 peptides) and chymotrypsin (1 peptide) digests of whole beer (Table 1).

We also examined the fragments identified in the undigested filtrate (<10 kDa) for peptides that shared homology with the known immunotoxic epitopes, as reviewed in Sollid et al.[42] It should be noted that only three of the reported epitopes[42] are specific to barley, and in our comparison, we considered the native version of the peptide sequence, i.e. without deamidation. Moreover, finding peptides with homologous sequences does not prove immunotoxicity; however, the persistence of these highly similar sequences in beers after PEP treatment could purport that known immunotoxic sequences are present but evaded our detection strategy. No peptide fragments comprising immunotoxic epitopes were detected in the <10 kDa fraction of the gluten-reduced beers LG1-LG4, LG8-LG9, and LG11-LG12. One to three homologous peptides were identified in the remaining beers (two in LG5 and LG6, three in LG7, and one in LG10). Figure 3 shows the alignment of three gluten-derived fragments with known immunotoxic sequences. Of the three, one of the peptides matched 8/9 amino acids with an L−Q amino acid substitution in the ninth position. It should be noted that the second fragment (QQAELIIP$^{\downarrow}$QQP$^{\downarrow}$QQP$^{\downarrow}$), while comprising two missed PEP cleavages, was cleaved within the QQP$^{\downarrow}$FP epitope, thus rendering this fragment invisible to ELISA and potentially detoxifying the fragment. A more diverse range of potentially immunotoxic peptide fragments was detected in both the undigested and digested control beers with several of these fragments showing exact matches to known immunotoxic epitopes. For example, a γ-gliadin fragment IIQPQQPAQLE-GIR was detected in the wheat beer C2 where the underlined sequence matches precisely to the epitope reported in two previous studies.[43,44]

Relative Quantitation of Gluten in Whole Beers. The tryptic and chymotryptic digested whole beers were then subjected to relative quantitation to examine the relative abundance of the gluten-derived peptides in the selection of

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Table 2. Analysis of Gluten Fragments Detected in the Sub-10 kDa Fraction of Control (C) and Low Gluten (LG) Beers (No Enzymatic Digestion)[a]

		N-terminus		C-terminus							
beer	gluten peptides[b]	cleavage at P−X	cleavage at X−P	cleavage at P−X	cleavage at X−P	% cleaved at P−X	% cleaved at X−P	% cleaved at X−P−X	missed cleavages (P−X)	number of P−X in peptides	% peptides with P−X
C1	22	1	0	3	1	9.1	2.3	11.4	50	0−5	90.9
C2	49	0	4	6	0	6.1	4.1	10.2	90	0−7	93.9
C3	58	1	3	12	2	11.2	4.3	15.5	113	0−5	91.4
C4	21	1	1	1	0	4.8	2.4	7.1	49	0−5	95.2
LG1	28	21	0	19	0	71.4	0.0	71.4	20	0−2	60.7
LG2	22	12	0	12	1	54.5	2.3	56.8	26	0−2	86.4
LG3	33	23	0	19	4	63.6	6.1	69.7	47	0−3	93.9
LG4	30	23	0	20	0	71.7	0.0	71.7	33	0−3	76.7
LG5	40	31	0	16	7	58.8	8.8	67.5	42	0−3	70.0
LG6	57	38	0	24	6	54.4	5.3	59.6	84	0−3	84.2
LG7	53	30	0	25	6	51.9	5.7	57.5	70	0−5	77.4
LG8	29	1	0	1	2	3.4	3.4	6.9	38	0−3	82.8
LG9	30	26	0	16	2	70.0	3.3	73.3	27	0−3	66.7
LG10	22	18	0	14	0	72.7	0.0	72.7	17	0−2	63.6
LG11	19	14	0	12	0	68.4	0.0	68.4	9	0−2	42.1
LG12	7	0	0	0	0	0.0	0.0	0.0	5	0−2	57.1

[a]The number of termini cleaved at the high affinity P−X or low affinity X−P sites are listed and converted to a percentage of total termini. Analysis of the number of missed cleavages (at P−X) within the gluten peptides is presented. [b]Gluten peptide fragments detected with >95% confidence.

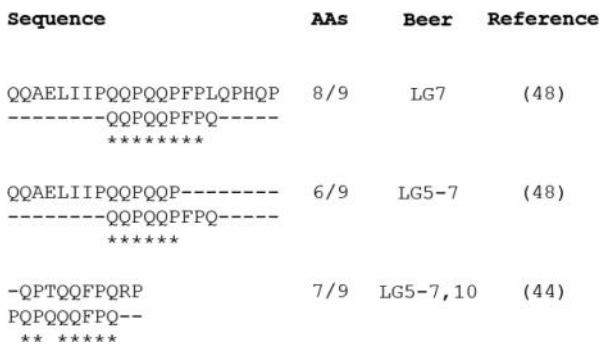

Figure 3. Potentially immunotoxic gluten fragments in sub-10 kDa filtrates. The peptide fragment detected is the top line with the number of matching amino acids in parentheses and the beers in which the peptide was detected. The immunotoxic epitope is indicated below with the reference to the study wherein the epitope was defined.

low gluten beers and in comparison to the control beers (Figures 4 and 5 respectively). LC-MRM-MS analysis of selected gluten-derived peptides is presented as the summed MRM peak area, which gives an indication of the peptide abundance. However, the MS response is peptide-dependent; the precise sequence is one of the determinants of the ionization potential, and the resultant peak area (and/or intensity). Consequently, the peak areas cannot be compared between peptides, but the peak areas may be compared between samples. While the approach employed does not allow determination of the absolute quantity of gluten in each beer, the control beers were previously analyzed by ELISA and yielded values of 227 mg/kg (C1), 5263 mg/kg (C2), 0.25 mg/kg (C3), and 1.1 mg/kg (C4).[45] The control beer C2 was a wheat beer, thus explaining the high value obtained. The remaining three control beers yielded ELISA results spanning three orders of magnitude. Comparing the LC−MS results of C3 (0.25 mg/kg) relative to those of C1 (227 mg/kg), it was noted that 10 of the 14 peptides monitored were detected with greater abundance in C3 after trypsin digestion (Figure 4). The peak areas for all 14 peptides were summed and revealed a 2.3-fold increased abundance in C3 relative to C1, which contrasts the results obtained by ELISA, wherein C1 contained ∼200-fold more gluten than C3. The lack of correlation between the LC−MS results and the ELISA data precludes an estimation of the gluten content of the gluten-reduced beers, and as such, the data are presented as raw peak areas.

Figure 4 shows the MRM peak area for 14 tryptic peptides selected to cover the range of gluten proteins detected. A peptide from an avenin-like protein (ALP, Uniprot: F2EGD5) was detected in relatively high abundance in both LG1 and LG8 (Figure 4A). In fact, the levels were as high as those noted in the control beers. Likewise, a peptide derived from a second ALP (Uniprot: M0VKM6) was noted to be 3−4 times higher in LG3−LG5 and LG7 (Figure 4B). The suite of B-hordeins expected in any given beer is dependent on the source barley cultivar, typically with approximately 10 B-hordeins being present in any single cultivar[46] and with heterogeneity in their composition in terms of both the isoforms present and their abundance. We monitored four B-hordein peptides that could represent a minimum of three B-hordein isoforms, although each peptide could be mapped to a different number of barley isoforms in the Uniprot database (18, 11, 24, and 1, respectively for Figures 4C−F). The three B-hordein isoforms monitored showed a consistent pattern with higher levels in LG7 and LG8 followed by LG3−LG5 (Figures 4C−F). Four peptides derived from the single D-hordein were monitored and detected across the range of low gluten beers with the exception of LG12, an expected result given this beer was made from barley devoid of D-hordein. Very low levels of the D-hordein peptides were detected in LG11. By monitoring multiple peptides from different regions of the protein, it is possible to investigate the degradation of the protein during the brewing process. Three of the four D-hordein derived peptides showed similar abundance patterns across the LG beers. The first and fourth peptides (ELQESSLEACR, Figure 4G and LEGGGGLLASQ, Figure 4J) were devoid of Pro (P). The second peptide was derived from a region that persists despite containing a potential PEP cleavage

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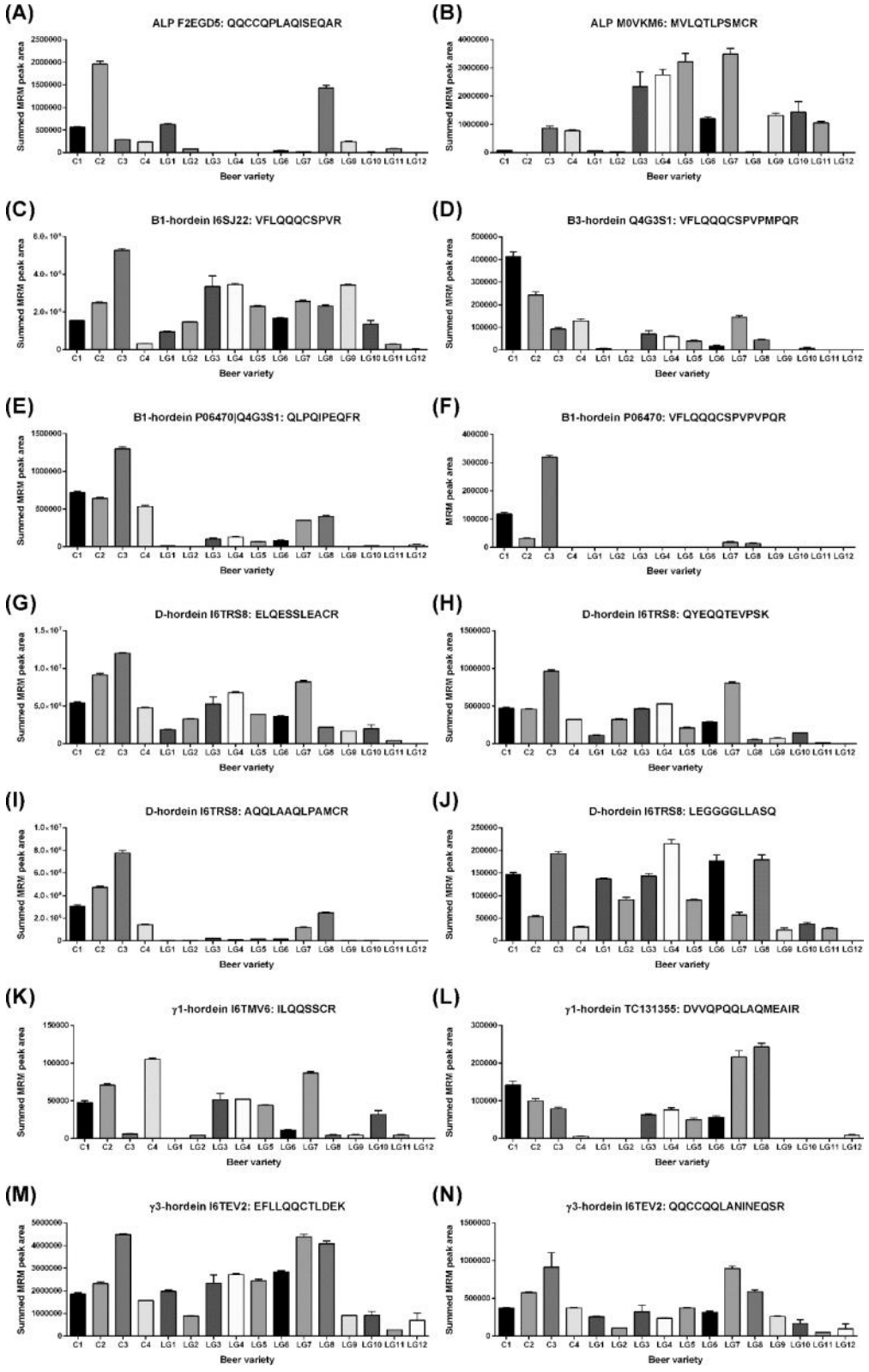

Figure 4. LC−MS analysis of tryptic peptides derived from gluten proteins in four commercial control beers (C1−C4) and 12 low gluten beers (LG1−LG12).

site (QYEQQTEVP$^{\downarrow}$SK, Figure 4H). The third D-hordein peptide monitored (AQQLAAQLP$^{\downarrow}$AMCR, Figure 4I) contained a single PEP cleavage site and was detected in high levels in the control beers (C1−C4) that were not subjected to PEP treatment, and in LG8, a beer processed without PEP treatment. This peptide was also detected in LG7 and to a lesser extent in LG3-LG6. These data imply that PEP cleavage is variable with some sites remaining uncleaved, possibly due to steric hindrance. The peptides originating from the N-terminal region of D-hordein showed a highly similar pattern of peptide

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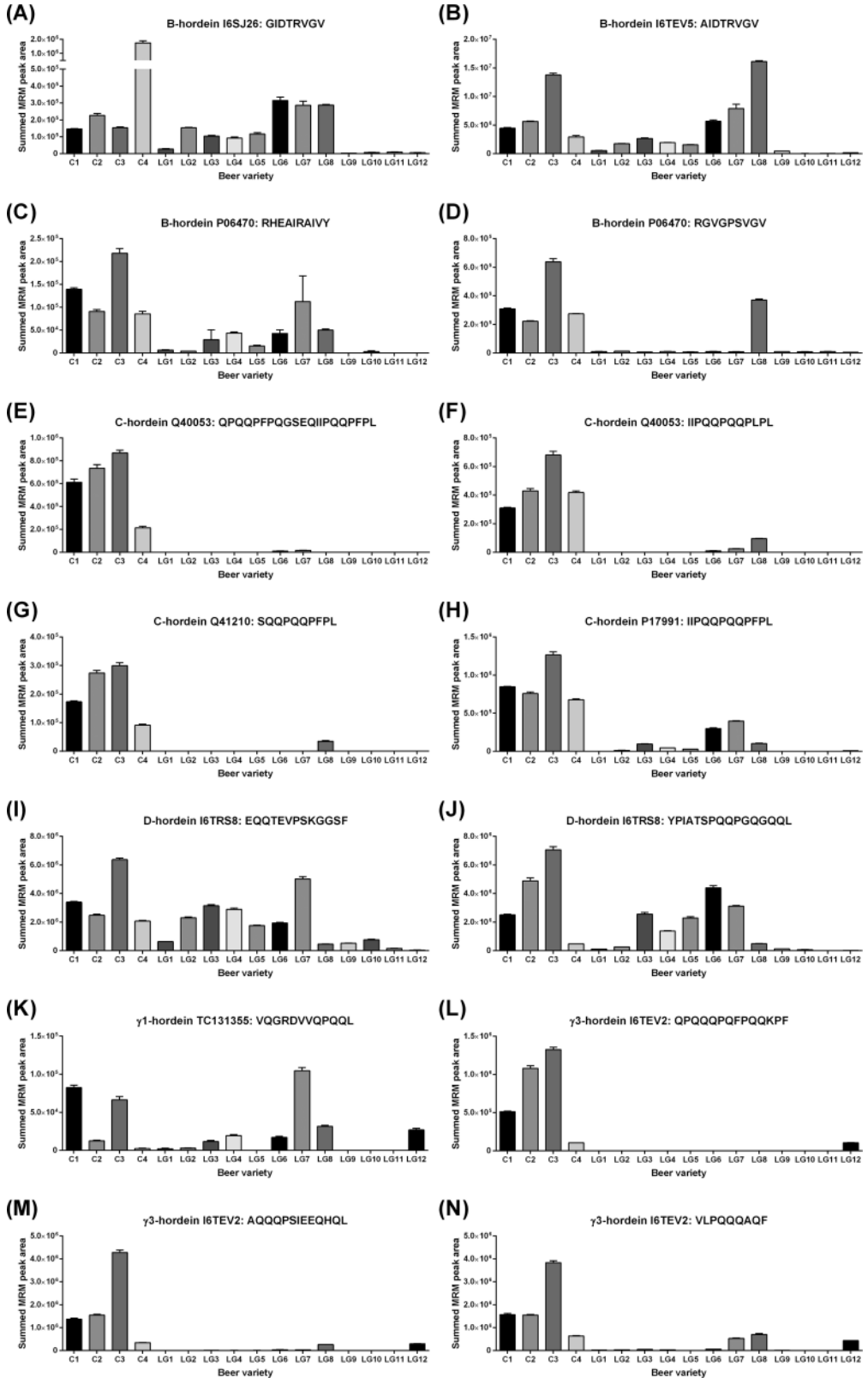

Figure 5. LC−MS analysis of chymotryptic peptides derived from gluten proteins in four commercial control beers (C1−C4) and 12 low gluten beers (LG1−LG12).

abundance (Figures 4G and H), and the pattern of peptide abundance was markedly different for the peptides from the C-terminal region of D-hordein (Figures 4I and J) which were different from each other. The persistence of the N-terminal peptides implies that this region of D-hordein is more resistant to the action of PEP. Examining the γ-hordeins, the levels were again noted to be generally higher in LG3-LG6 and LG7-LG8. Only one (DVVQP$^{\downarrow}$QQLAQMEAIR, Figure 4L) of the four γ-

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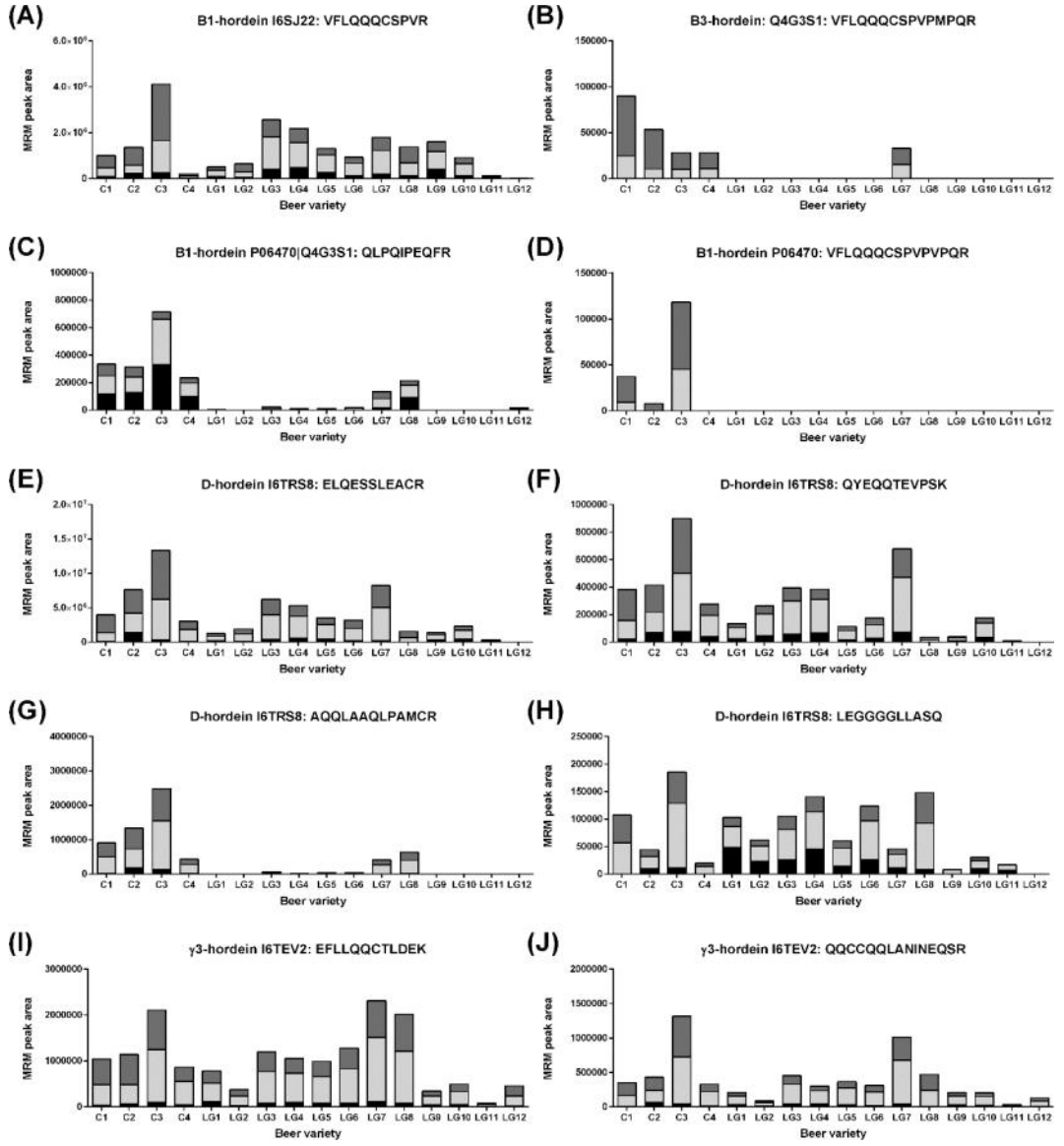

Figure 6. LC−MS analysis of tryptic peptides derived from gluten proteins after size fractionation in four commercial control beers (C1−C4) and 12 low gluten beers (LG1−LG12). The columns represent the different size fractions: >30 kDa (dark gray); 10−30 kDa (light gray); and <10 kDa (black).

hordein peptides contained a PEP cleavage site. Despite use of PEP in the production of LG3−LG7, notable levels of this peptide remained, demonstrating that the PEP treatment applied in the production of these beers was not efficient in hydrolyzing all potential sites. The level of this peptide was lower in other PEP treated beers, LG1 and LG2, and LG10 and LG11. The incomplete digestion of these peptides by PEP in all beers is consistent with the data from the analysis of the <10 kDa fraction, which also revealed incomplete cleavage by PEP (Table 2). The different levels of peptides that contain cleavage sites in the gluten-reduced beers suggest that some brewers have optimized the digestion process to a greater extent than others. However, complete digestion was not observed in any of the samples, suggesting that PEP as it was applied does not completely digest all gluten fragments during the brewing process.

The use of chymotrypsin is complementary to the use of trypsin. We monitored three B-hordein isoforms; the first two (I6SJ26, Figure 5A and I6TEV5, Figure 5B) were not assessed using trypsin but showed a similar trend in that the gluten-reduced beers LG6−LG8 revealed the highest abundance, and very low levels were detected in LG1and LG9−LG12. The third B-hordein isoform (P06470, Figures 5C and D) was also tracked using trypsin (Figures 4E and F). The pattern of abundance was consistent across the control beers (C3 > C1 > C2 ∼ C4), but there were some variations noted across the gluten-reduced beers. Despite this, LG7 and LG8 showed the highest levels with lower levels noted in LG3−LG6. The C-hordeins contain few tryptic cleavage sites and as such are under-represented when sample preparation (protein digestion) is conducted using trypsin. The C-hordeins are thought to precipitate out during malting and brewing. Despite this, we followed three C-hordein isoforms by LC-MRM-MS (Figures 5E−H). All three isoforms were detected in the control beers (C1−C4) and were additionally detected in LG8, a beer produced without the use of PEP (12−21% relative to the control beer average). The C-hordeins are the most notable immunopathogenic proteins in barley[47] and were decreased by

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the greatest extent after PEP treatment, which can be correlated with the higher proportion of Pro in C-hordeins (~30%) compared to the other hordein proteins (range 10−20%). However, a few of the LG beers were noted to contain low levels of the C-hordeins. The C-hordein isoform (Q40053) was detected by two peptides in LG6 (1.8 and 2.2%) and LG7 (2.7 and 5.4%) (Figures 5E and 4F, respectively). A single peptide derived from the C-hordein isoform (P17991) was detected in LG3 (10.9%), LG6 (33.6%), LG7 (44.8%), and at low levels (<5%) in LG2, LG4-LG5, and LG12 (Figure 5H). The detected peptide species were also compared to the known immunotoxic epitopes[42] wherein two of the C-hordein (from Q41210 and P17991) peptides comprising known epitopes were detected in the control beers (C1−C4). While there were no precise matches to the nonapeptide epitopes, three of the C-hordein sequences (Figures 5E, G, and H) contained 8/9 matching amino acids,[48] as indicated by underlined region of peptide sequences that follow. The level of these peptides were not insignificant with ~17% (relative to the average control beer content) of the C-hordein peptide SQQPQQPFPL in LG8, and varying levels of IIPQQPQQPFPL in beers LG2−LG7 (2−45%) and LG8 (12%). Three of the C-hordein peptides detected in the MRM analysis of the gluten-reduced beers contained the QQPFP antigenic epitope, but these were not identified directly in the LG beers using data-dependent acquisition in the discovery phase. This is not unexpected given the stochastic nature of ion sampling and highlights the increased sensitivity of targeted MS analysis. Reviewing the chymotryptic peptide sequences, those devoid of PEP cleavage motifs (P−X or X−P as shown in Figures 5A−C) generally yielded higher levels in the LG beers, while those containing proline (Figures 5D−H and L−N) typically showed lower levels in the PEP-treated LG beers. One exception was the D-hordein derived sequence EQQTEVP$^{\downarrow}$SKGGSF (Figure 5I), which was observed at levels comparable to the control beers in LG2−LG7 and at lower levels in LG1 and LG9−LG11. This correlated with that observed for the same region (QY**EQQTEVPSK**GGSF) of the protein after tryptic digestion (Figure 4H), wherein the bold font represents the overlapped region between the tryptic and chymotryptic peptides monitored, and demonstrated that enzymatic hydrolysis by PEP is not an efficient process. Likewise, the D-hordein peptide YP$^{\downarrow}$IATSP$^{\downarrow}$QQP$^{\downarrow}$GQGQQL contains three missed PEP cleavage sites (Figure 5J). Three of the four γ-hordein peptides contain a single PEP site (Figures 5K and M−N) and were highest in abundance in LG7 and/or LG8. Notably, the γ3-hordein peptide containing three P−X sites (Figure 5L) was absent in all but LG12, wherein this peptide was expected based on the presence of I6TEV2 in the barley malt used to produce the beer.

Relative Quantitation of Gluten in Size Fractionated Beers. The beers were sequentially passed through 30 kDa and 10 kDa MWCO filters aimed at a crude size-based fractionation of the proteins. The retained protein or filtrate for the sub-10 kDa fraction were then digested with trypsin. Figure 6 shows the proportion of the signal as detected in the three size fractions. The pattern of peptide abundance noted in the on-filter digestion was highly similar to that observed after in-solution digestion of whole beer with trypsin (Figures 4C−J, M, and N). The B- and γ-hordeins were detected in both the 10−30 kDa and >30 kDa fractions, which was not a surprising result given that their expected MW were in the range 28−33 kDa. The D-hordein was most abundantly detected in the 10−30 kDa fraction (~50% of D-hordein peptide signal present, Figures 6E−H) despite having an expected MW of 75.0 kDa, implying that hydrolysis of the protein had occurred, but this was not significantly different to that observed for the control beers. The only notable difference was in a greater proportion of the C-terminal peptide (Figure 6H: LEGGGGLLASQ) present in the <10 kDa fraction in the LG beers (range 6−47%, median 28%) compared to the control beers (range 0−23%, median 3%), indicating that hydrolysis in the C-terminal region of the D-hordein had liberated a C-terminal fragment of size <10 kDa. Although variable depending on which gluten peptide was monitored, an average (considering all peptides in Figure 6) of only ~6% of the tryptic peptides were detected in the <10 kDa fraction, and ~94% was derived from protein fragments >10 kDa (34% as 10−30 kDa and 60% as >30 kDa) or 90 amino acids in length (assuming an average MW of 110 Da per AA). The obvious exception to this was the C-terminal D-hordein peptide (Figure 6H) that gave averages of 23% (<10 kDa), 53% (10−30 kDa), and 24% (>30 kDa).

The results presented here demonstrate that while the addition of PEP during the brewing process reduced the gluten content of beer, the digestion was not complete in any of the PEP-treated beers analyzed, with many missed cleavage sites detected. However, in some instances (LG11), the treatment with PEP was effective in reducing the gluten to very low levels, and the gluten peptides that were detected contained a lesser proportion of missed cleavage peptides. This analysis represents a snapshot in time with only one batch of each beer tested, but because of the incomplete nature of the treatment, it is possible that different peptides and relative amounts of these peptides would be observed. The presence of peptides with potentially immunotoxic epitopes, and those containing the sequence QQPFP is in partial agreement with a recent analysis of AN-PEP-treated beers, which did not identify any peptides containing the sequence QQPFP in AN-PEP treated beers[31] as only a single peptide containing QQPFP was detected in the undigested filtrate of LG7 in the preliminary discovery proteomics experiments (Supplementary Table 1), thus explaining why the gluten in these beers remains invisible to ELISA determination. However, when the samples were analyzed by MRM-MS, three C-hordein derived peptides containing the QQPFP motif were detected in the gluten-reduced beers in addition to the control beers. This difference between the two studies may be in part due to our study including analysis of a greater range of commercial beers and using targeted proteomics, which provides greater sensitivity than data-dependent acquisition strategies. The presence of these peptides at different levels in the gluten-reduced beers analyzed in this study is consistent with the recent finding that sera from some people with CD reacted to some gluten-reduced beers.[18]

In this study, we demonstrated that in beers treated to remove/reduce gluten that are <20 mg/kg as measured using ELISA by the beer manufacturer, detectable levels of gluten peptide fragments remain. These peptides span the length of many of their protein precursors and moreover are present in fragments presumably larger than 10 kDa or 90 amino acids as judged by analysis after size fractionation. The examination of filtered (undigested) beers clearly revealed an increased frequency of hydrolysis of the protein backbone at P−X motifs in those beers treated with a prolyl endopeptidase. However, the efficiency of cleavage at proline residues remains in question with many motifs showing resistance to the enzyme.

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The presence of large protein fragments in the gluten-reduced beers after PEP treatment and/or proprietary processing are a cause for concern for those people with celiac disease as they may contain sufficient immunopathogenic sequences to elicit adverse reactions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b03742.

Supplementary Table 1: Gluten peptide detected at 95% confidence in control (C1−C4) and low gluten (LG) beers after trypsin digestion (A), chymotrypsin digestion (B) or undigested filtrate <10 kDa (C) (PDF)

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Notes

The authors declare no competing financial interest.

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