AN-PEP, Proline-Specific Endopeptidase, Degrades All Known Immunostimulatory Gluten Peptides in Beer Made from Barley Malt

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ABSTRACT

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The haze-forming peptides and proteins present in beer mainly originate from the grains used. Such grains, typically barley and wheat, also incorporate gluten. Although partially hydrolyzed during brewing, gluten-containing protein material remains present in the final beers. Gluten-sensitive people might therefore not be able to consume beer without an adverse response. AN-PEP, the proline-specific peptidase in Brewers Clarex, is commercially applied to selectively degrade proline-rich, haze-forming peptides and proteins during beer fermentation. The unusually high percentage of proline in known celiac-related gluten epitopes suggests that they form ideal substrates for AN-PEP as well. We confirmed that in beers prepared with AN-PEP, gluten levels were well below 20 mg/kg based on the established competitive R5 ELISA method. We used a sensitive LC-MS/MS method developed in-house to identify the peptides. Only in beers brewed through a conventional process could we find epitope-containing peptides, even in beers that contained less than 20 mg/kg of gluten based on the ELISA. So even though the MS method is qualitative and not quantitative, it provided a more detailed view of the fate of gluten during brewing. Specifically, these data indicate that AN-PEP is able to degrade all known immunogenic gluten epitopes in beer. We conclude that apart from its current application as a beer stabilizer, AN-PEP has the additional benefit of exhaustive degradation of all potentially problematic gluten epitopes.

Keywords: AN-PEP, Brewers Clarex, ELISA, Gluten, Mass spectrometry

Gluten is best known for giving texture and elasticity to the dough of wheat bread, but it is commonly present in a large variety of foods (25,34,40,58,66). Approximately 60% of the protein content of the *Triticum* grains wheat, barley, and rye consists of the seed storage proteins collectively named gluten (54). Gluten is subdivided into two main protein families: glutelins and prolamins, originally categorized as such based on their alcohol solubility. Wheat contains three related genomes, containing 20–30 glutenin genes and on the order of 100 gliadin genes, across nine loci, which together make up the gluten component of the grain (56). In barley, the gluten-like proteins are termed hordeins. There are four hordein protein families, the B-, C-, D-, and γ-hordeins, of which B- and C-hordeins also comprise multigene families. Both glutelins and prolamins contain unusually high amounts of proline (≈20%) and glutamine (≈38%) residues. These occur in repetitive stretches in the primary sequence and are highly resistant to gastrointestinal peptidases. Thus, relatively large, proline-rich protein fragments can reach the duodenum and proximal small intestine upon the oral intake of gluten. There, upon activation by human tissue transglutaminase (tTG), these gluten peptides can stimulate the human leukocyte antigen (HLA) HLA-DQ2/8 (restricted gluten-reactive $CD4^+$ T-cells in individuals suffering from celiac disease), ultimately leading to chronic inflammation of the intestinal mucosa and subsequent nutrient malabsorption (33,35,36,38,43,59,60,62,72,74). Grain species other than barley, wheat, or rye contain similar storage proteins not enriched in so much proline, and these are not regarded as immunogenic (53). It is estimated that at least 1% of the Western world population is afflicted by celiac disease (25). Not only celiac patients but also nonceliac individuals can experience physical problems upon gluten ingestion. Recently, this so-called nonceliac gluten sensitivity (or gluten sensitivity) has been clinically recognized as a separate condition (21). Although the symptoms experienced by such gluten-sensitive subjects are often identical to those seen in celiac disease, it has not been determined yet which grain components are responsible.

With no treatment available, gluten-intolerant people are required to follow a strict lifelong gluten-free diet. Foods with gluten levels <20 mg/kg are generally regarded as gluten-free. The Codex Alimentarius recommends the R5 enzyme-linked immunoassay (ELISA) to measure gluten, whereby the monoclonal R5 antibody (2,31,49) recognizes the peptide motifs QQQFP, LQPFP, QLPFP, and the common repetitive QQPFP motif found in all immunogenic prolamins. Sandwich ELISA requires detection of two R5 epitopes per protein fragment (16,64). In products that contain hydrolyzed protein, such as beer, small remaining hordein peptides may not necessarily contain two R5 epitopes. Therefore, for such products a competitive ELISA setup is recommended (10,13,14). The main disadvantage of these or any antibody-based assays is that they will only detect proteins and protein fragments containing the motifs specific for the antibody used. Consequently, proteins or peptides lacking these specific motifs are missed. On the other hand, an antibody may bind to small peptides containing one of the aforementioned motifs, even though it is known that the immunogenic peptides causing problems in celiac patients require a minimum length of nine amino acids (3,8,30,32,37,39,58,70). It has been shown that quantitation of ELISA is only possible if a protein standard comparable to the protein being measured is used (62,63). The RIDASCREEN kit (recently validated for detection of gluten in beer; R-Biopharm, Darmstadt, Germany) uses a hydrolysate of wheat, rye, and barley as a reference.

Beer brewing is the oldest biotechnological process known to humans, whereby beer is produced via fermentation of malted barley (*Hordeum vulgare)* extract and to a lesser extent of malted wheat (*Triticum aestivum*) extract. Although most of the grain proteins in beer are removed during wort boiling and cooling, several proteins and protein fragments remain in the finished product. An average beer has a protein content of around 500 mg/L (12,52), incorporating a limited number of intact proteins and

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larger protein fragments such as serpin-Z4, lipid transfer protein 1, and some proline-rich peptides (6) originating mainly from hordeins. To guarantee a clear, shelf-stable beer, removal of these proline-rich haze-sensitive peptides is of paramount importance, because they can associate with beer polyphenols to create insoluble aggregates (6,45). Conventionally, this is achieved via polyvinylpolypyrrolidone (PVPP) or silica treatment. However, these treatments do not usually remove all proline-rich immunogenic gluten peptides, leaving these beers unsuitable for consumption by gluten-intolerant people (28,45). A proline-specific endopeptidase AN-PEP from *Aspergillus niger*, commercially available as Brewers Clarex, is frequently used for beer stabilization in the brewery industry (7,15,20,41). AN-PEP hydrolyses proteins at the carboxylic end of proline residues (19,46,48,61,63), thereby degrading the haze-sensitive peptides and proteins into smaller peptide fragments that can no longer associate with polyphenols to form haze (41). The enzyme has its pH optimum around 4.5. This matches with the pH of the fermenting beer, allowing application of the enzyme without making any pH adjustments. Thus, addition of AN-PEP during beer fermentation effectively prevents chill-haze formation while leaving foam stability and flavor intact (41).

Separately, a number of in vitro and clinical studies have shown the complete degradation of wheat-derived, proline-rich, immunogenic gluten peptides by AN-PEP under gastric conditions (29,46,61). Therefore, it is anticipated that AN-PEP will hydrolyze barley- and wheat-derived proline-rich immunogenic gluten peptides during acid beer fermentation conditions as well, offering a unique tool for producing beers acceptable for gluten-intolerant people.

Recently, liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been explored as a nonimmunological alternative for detection of trace levels of gluten in beer (11,12,23,52, 65,76). The main advantage of this technique is that, in contrast to using ELISA techniques, epitopes are identified purely on the basis of their amino acid sequence, thus providing an unbiased view of the beer proteome and peptidome (65).

In this study, we investigated the effects of AN-PEP on gluten epitope degradation in beer. In AN-PEP treated beers, competitive R5 ELISA failed to detect any gluten and, impressively, also when using LC-MS/MS no gluten peptides that are considered potentially problematic for gluten-intolerant people could be detected. Therefore, the current application of AN-PEP in the brewing process as a stabilizer has the degradation of all known immunogenic gluten peptides as an additional benefit.

EXPERIMENTAL

Sample Selection and ELISA Procedure

The sample set consisted of five pairs of beers produced in AN-PEP trials, in which the brew was split into two fractions before fermentation; one half was conventionally fermented, and to one half AN-PEP was added. This sample set was supplemented with two commercial gluten-free beers for which AN-PEP was used to degrade gluten. These two samples were accompanied by an analysis certificate stating that the gluten level was below the limit of quantification (LOQ) of the R5 competitive ELISA, as measured by a certified contract laboratory. Samples were analyzed using the RIDASCREEN Gliadin competitive ELISA (R7021, R-Biopharm) according to the provided instructions. The extraction was performed on degassed beer samples using a solution of 60% ethanol containing 10% fish gelatin (G7765, Sigma, St. Louis, MO, U.S.A.). The ELISA procedure was performed immediately after the extraction, and results were considered valid when the response was between 30 and 70% of the calibration curve, or when the response was below the LOQ while using the minimum sample dilution specified.

Sample Pretreatment for MS Analysis

Degassed beer samples were stored at 4°C. Three 100 µL aliquots were taken from each sample. Sample preparation was performed as described by Colgrave (11). Briefly, after reduction and alkylation the pH of the samples was checked to be ≈7.5. (Chymo-)trypsin (sequencing grade, Roche, Indianapolis, IN, U.S.A.) stock solutions were prepared in 0.01N HCl. To the aliquots 5 µL of either trypsin or chymotrypsin (1 µg/µL) was added followed by incubation for 5 h at 37°C. Then an extra 5 µL of 0.25 µg/µL peptidase was added followed by incubation overnight at 37°C. The third aliquot was not digested and was stored at 4°C overnight. Centrifugal devices with a 10 kDa filter (Pall, Port Washington, NY, U.S.A.) were washed with Milli-Q water. Samples were acidified with formic acid and filtered over the washed 10 kDa filters, and the filtrates were collected and injected on the LC-MS/MS system.

LC-MS/MS Analysis

The samples were analyzed on the Accela LTQ-Orbitrap LC-MS (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Peptides were separated on a C_{18} column (Zorbax Eclipse XDB-C18, 2.1 × 50 mm, 1.8 µm, and Poroshell 300SB-C3 narrow bore guard column, 2.1 × 12.5 mm, 5 µm, Agilent, Palo Alto, CA, U.S.A.). The column oven temperature was set to 50°C, and the injection volume was 20 µL. Mobile phase A was 0.1% formic acid in water (UHPLC grade, Biosolve, Valkenswaard, the Netherlands), and mobile phase B was 0.1% formic acid in acetonitrile (UHPLC grade, Biosolve). The gradient was run with a flow rate of 400 µL/min and went from 0 to 30% B in 70 min, followed by 5 min of flushing at 80% B and 5 min of equilibration at 0% B, for a total run time of 80 min. An *N*th order double play method was applied, measuring a mass range of 300–2,000 *m/z* with a resolution of 7,500 in the Orbitrap and MS/MS for the top five peptide ions from the MS scan in the linear ion trap. Dynamic exclusion was used with repeat count 2 and rejection time 10 s. Unassigned charge states were rejected, and all assigned charge states were included.

Database Searching

The LC-MS/MS results were searched with Sorcerer 2 (SageN, Milpitas, CA, U.S.A.) and SEQUEST (version 27, rev. 11, Thermo Fisher Scientific) search algorithms. First, the trypsin fractions were searched against the UniProtKB/Swiss-Prot database (date 4/23/2013, with trypsin cleavage preferences) to check the origin of the identified peptides. The precursor mass tolerance was set to 5 ppm, and no static modifications were considered; methionine oxidation, deamidation of glutamine and asparagine, and cysteine carbamidomethylation were allowed as variable modifications. Further critical parameters are described in the Discussion section. Trans-Proteomic Pipeline data analysis software was used, and only proteins with protein probability >0.9 were considered. Proteins from *H. vulgare* and *S. cerevisiae* were identified; therefore, an extracted database from the UniProtKB/Swiss-Prot database was created containing the proteomes of these organisms. The sequences of chymotrypsin, trypsin, and AN-PEP were added. The database was processed with a "no enzyme" setting, in the mass range 400–4,000 Da. The complete LC-MS/MS dataset was searched against this database. Results were exported to Excel and combined into one file, with protein probability >0.9, peptide probability >0.3, and protein groups hidden. The results were visualized with Spotfire software (Tibco, Boston, MA, U.S.A.).

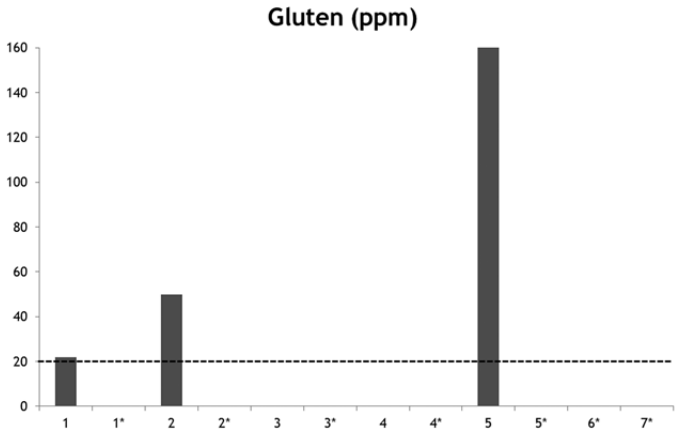

Fig. 1. The gluten content of different types of beer measured with the competitive R5 ELISA. An asterisk (*) indicates AN-PEP treatment. The gluten-free threshold (20 mg/kg) is indicated by the dotted line.

RESULTS

Gluten Levels in Conventionally Processed Versus AN-PEP Treated Beers

As shown previously (11,12,44,77), beers can vary greatly in their gluten content, and that level can be influenced by AN-PEP (26,42,75). To study in depth the effect that application of AN-PEP has, we selected samples from six different breweries. In five cases, these consisted of couples of beers in which the wort was split into two batches prior to fermentation: one batch was fermented and processed in a conventional way, that is, without adding AN-PEP but using either PVPP or silica to achieve stabilization. To the other batch AN-PEP was added at the start of fermentation, and these beers were further processed without using the classical clarification procedures. The other two beers were commercially available gluten-free barley-based beers in which AN-PEP was used during fermentation. To quantify the gluten, we used the ELISA setup as advised by the Codex Alimentarius: a competitive setup based on the R5 antibody. Whereas

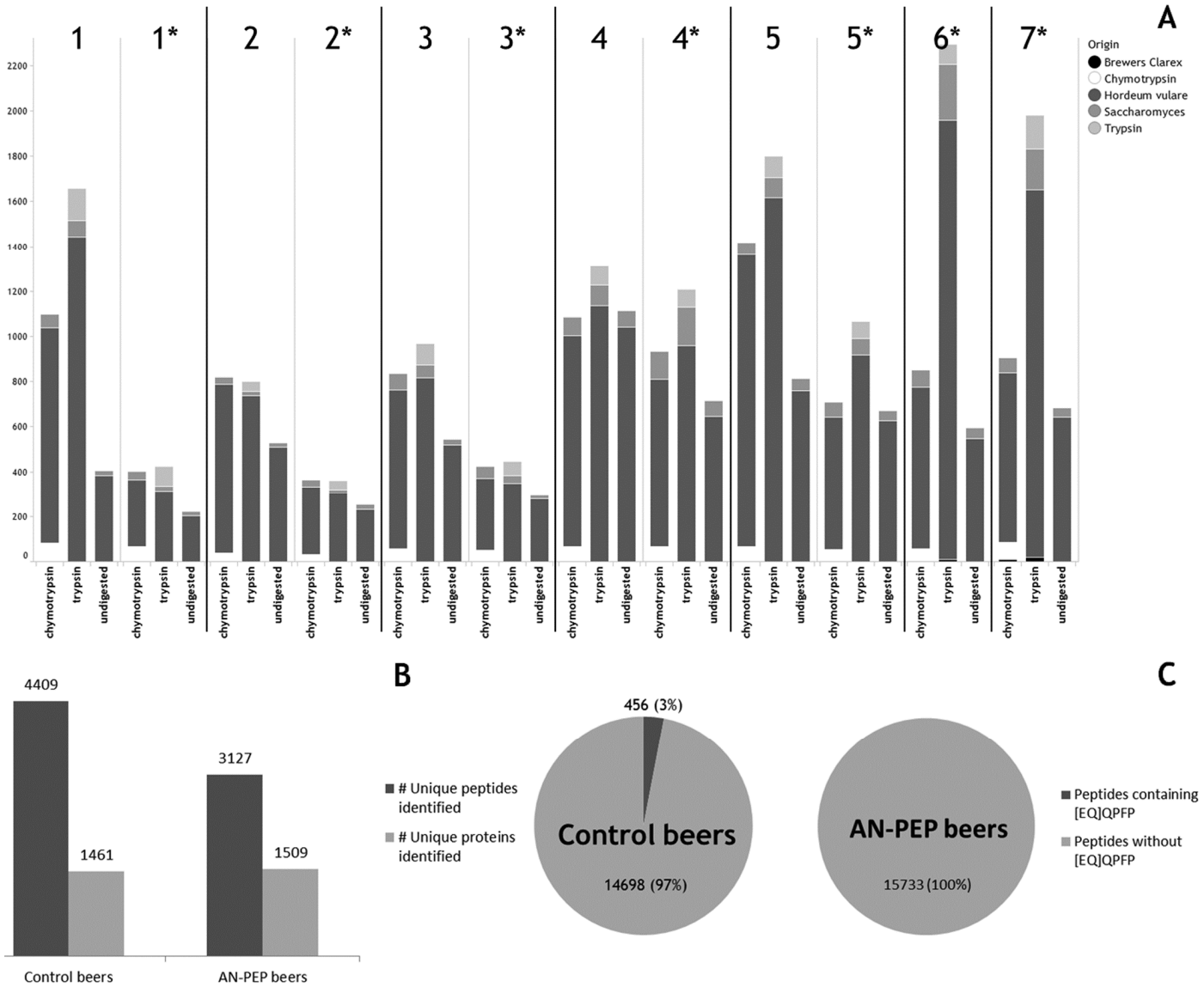

Fig. 2. LC-MS/MS results. **A,** The bars represent the total number of spectra matched to a peptide sequence in each of the aliquots. The shades of grey indicate the origin of the identified peptides. Samples 1–5 are conventional and AN-PEP treated beers compared per brewery; 6 and 7 are commercial beers brewed with AN-PEP; and an asterisk (*) indicates AN-PEP treatment. **B,** Total number of unique peptides and proteins identified in the control beers and the AN-PEP treated beers. **C,** Number of peptides identified; peptides containing potential epitopes are specified.

gluten levels of the conventionally processed beers were found to vary between 160 and below 10 mg/kg, gluten levels of the AN-PEP treated beers were all below 10 mg/kg according to the competitive R5 ELISA (Fig. 1). These observations led us to ask whether there still were gluten epitopes present in the beers that were below 10 mg/kg in ELISA, and specifically whether we could detect differences between the couples of beers (numbers 3 and 4) for which the control samples also showed undetectable gluten in ELISA. This required an approach that provided sequence information on the amino acid level. Therefore, we set up a nonquantitative LC-MS/MS method that allowed us to look in an unbiased way in molecular detail at both intact proteins and protein fragments in beer.

LC-MS/MS Experimental Setup

During the brewing process, part of the protein in wort will be hydrolyzed, giving rise to a large range in molecular weight (MW) of the protein material in beer (6,22,51,79). To analyze in an unbiased way, we used three different sample preparations: without predigestion to study the low MW material, and with predigestion by either trypsin or chymotrypsin to study the medium to high MW material. The various aliquots were then analyzed with LC-MS/MS using a top five data-dependent method. With this method, the MS recorded a mass spectrum and determined the top five peptide ions for which fragmentation MS/MS spectra were recorded. First, we identified the source of the proteinaceous material by searching the trypsin-treated aliquots against the complete UniProtKB/Swiss-Prot database, using the Sorcerer 2–SEQUEST search engine. We could confirm that the proteins and peptides stemmed from either *H. vulgare* or *Saccharomyces cerevisiae*. Next, a custom database was constructed containing the publically known protein sequences of *H. vulgare* and *S. cerevisiae* plus the protein sequence data of trypsin, chymotrypsin, and AN-PEP. Subsequently, all data from trypsin, chymotrypsin, and untreated fractions were searched against this custom database, using a "no enzyme" database search because beer represents a random protein hydrolysate. Finally, this search resulted in a data set of roughly 31,000 peptide identifications (Supplementary Table I).

The total number of identified peptides with a barley or yeast origin in each of the three aliquots is shown in Figure 2A. In the conventionally processed beers, 4,409 unique peptides originating from 1,461 unique proteins were identified (Fig. 2B). In beers that were treated with AN-PEP, 3,127 unique peptides originating from 1,509 unique proteins were identified (Fig. 2B). As expected, the majority of the identified peptides originated from

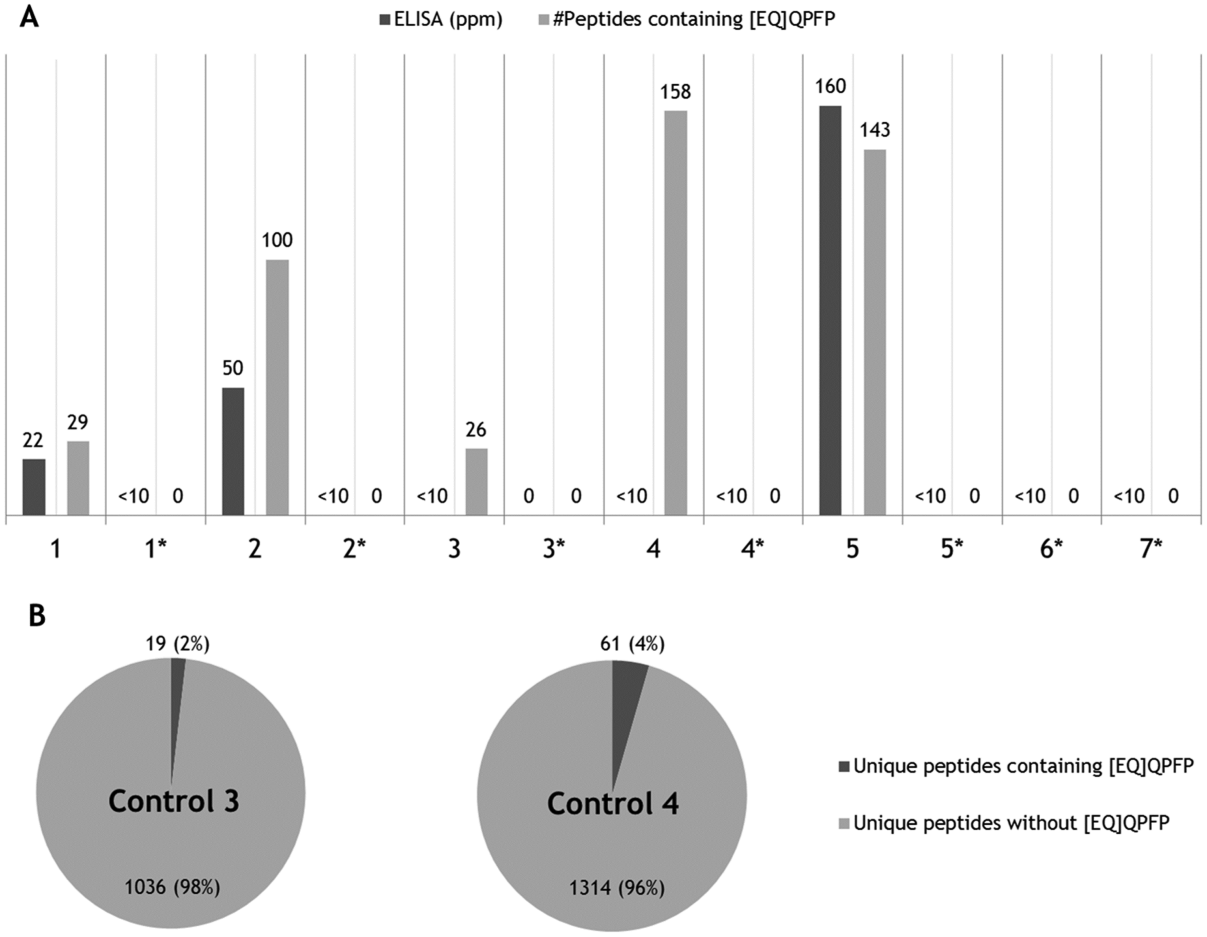

Fig. 3. A, The ELISA scores and LC-MS/MS identified [EQ]QPFP epitope containing peptides compared. The dark bars to the left represent the ELISA score in mg/kg, and the light bars to the right represent the total number of identified [EQ]QPFP containing peptides. An asterisk (*) indicates AN-PEP treatment. No [EQ]QPFP peptides were identified in any of the beers brewed with AN-PEP, and their ELISA score was below the detection limit of 10 mg/kg. Three conventionally processed beers (control 1, 2, and 5) scored >20 mg/kg in ELISA, and various [EQ]QPFP containing peptides were identified with LC-MS/MS. Two conventionally processed beers (control 3 and 4) scored below the detection limit in ELISA, but significant numbers of [EQ]QPFP containing peptides were identified with LC-MS/MS. **B,** The percentage of unique [EQ]QPFP containing peptides identified in conventionally processed beers 3 and 4.

H. vulgare. The number of unique peptides identified in AN-PEP-treated beers was lower than in untreated beers. This was not surprising, because the additional proline-specific cuts introduced into the already hydrolyzed wort proteins would result in even smaller fragments that may have become too small for LC-MS/MS identification (the minimal length of identified peptides in the current experiment is five amino acids) or may have become redundant. Indeed, the average length of peptides identified in untreated aliquots was 13 amino acids in the control beers and 11 amino acids in AN-PEP treated beers.

Identifying Gluten Epitope-Containing Peptides

To focus on the gluten epitope-containing fragments in our dataset, we compiled an epitope list (Supplementary Table II) incorporating all currently known celiac disease relevant T-cell epitopes (4,57). Because deamidation of glutamine (Q) by tTG (effectively changing Q into E) can be a prerequisite for an immune response (5,57,67–69,73), the relevant hordein sequences expected after exposure to tTG activity were also added (Supplementary Table II [17,31,57,68,71]), to include the possibility that deamidated peptides were formed during brewing. Finally, this epitope list was complemented with the minimal core sequences QQPFP, EQPFP, QQQFP, QLPFP, and LQPFP known to be recognized by the R5 antibody (31). Of these five sequences, only [EQ]QPFP forms part of the known immunogenic gluten epitopes DQ2.5-glia-gamma5 and gamma4c. Therefore, we used this sequence as a marker to mine the complex LC-MS/MS dataset and to compare the identified peptide sequences with our R5 ELISA results. The data obtained indicated that no [EQ]QPFP containing peptides were present in the AN-PEP treated beers, whereas a significant proportion of such peptides could be identified in the conventionally processed control beers.

Known gluten epitope from Supplementary Table II → / Identified Sequence ↓	QQPQQPFPQ	QQPFPQQPQ	PFPQPQQPF	PQPQQPFPW	PFPQPQQPF	PQPQQPFPQ	PIPQQPQPY	PFPQPQQPF	PQPQQPFPQ	QQPQQPFPQ	QQFPQPQQPFPQQP	QQPFPQQPQQPFPQ	FPLQPQQP	#epitopes in peptide
FPLQPQQPFPQ													1	1
FPQPQQPFPW				1										1
FSFSQQPQQPFPLQPQQPFPQQPQQPFP		1											1	2
IPQQPQQPFPQQPQQP	1	1								1				3
IPTPLQPQQPFPQQPQQPLPRPQ		1												1
LQPQQPFPQQPQQP		1												1
LQPQQPFPQQPQQPLPQQP		1												1
PFPQQPQQPFPQP	1									1				2
PFSFSQQPQQPFPLQPQQP													1	1
PLQPQQPFPQQPQQPFPQPQQP	1	1								1		1		4
PQQPFPLQPQQPFPQ													1	1
PQQPFPLQPQQPQPF													1	1
PQQPFPQPQQPFPLQPQQP			1		1			1					1	4
PQQPFPQPQQPFPW			1	1	1			1						4
PQQPFPQQPQQPFPQP	1	1								1		1		4
PQQPQQPFPQQPQ	1	1								1				3
PQQPQQPFPQQPQQP	1	1								1				3
QPFPLQPQQPFPQQPQQP		1											1	2
QPFPQPQEPIPQQPQQPFPQQP	1									1				2
QPFPQQPQPFPQQPIPQQPQPY							1							1
QPFPQQPQPFPQQPIPQQPQPYP							1							1
QPFPQQPQPFPQQPIPQQPQPYPQQP							1							1
QPFPQQPQPFPQQPIPQQPQPYPQQPQP							1							1
QPFPQQPQQPFPQQ	1									1				2
QPQQPFPLQPQQPFPQQP													1	1
QPQQPFPQPQQPFPL			1		1			1						3
QQPFPQQPQLP		1												1
QQQPFPQQPIPQQPQPYPQ							1							1
SQQQFPQPQQPFPQ						1			1					2
SQQQFPQPQQPFPQQPQQPFPQ	1	1				1			1	1	1	1		7
Total epitope containing peptides: 30														
Total number of epitopes identified: 62														

Fig. 4. The identified gluten epitope-containing peptides in conventionally treated beer samples 3 and 4. The identified peptides are listed in the left-hand column, and the known immunogenic gluten epitopes these peptides contain are shown at the top. As shown, some of the identified peptides contain multiple epitopes. In total, 30 unique epitope-containing peptides were identified in the conventionally treated beer samples 3 and 4, which together contained 62 known gluten epitopes.

Comparing ELISA Results with LC-MS/MS Identifications

According to the R5 ELISA tests, none of the AN-PEP treated beers (Fig. 3A) showed any detectable gluten, whereas three of the five conventionally processed beers (beers 1, 2, and 5, Fig. 3A) contained detectable amounts of gluten and two (beers 3 and 4, Fig. 3A) contained <10 mg/kg. Our LC-MS/MS analysis showed, however, that all five conventionally processed beers contained many different [EQ]QPFP-containing peptides. The number of unique peptides containing [EQ]QPFP in each of the samples is shown in Figure 3A. Even in beers 3 and 4, which scored <10 mg/kg of gluten in the R5 ELISA test, such peptides were identified: 19 and 61 unique [EQ]QPFP-containing peptides, respectively (Fig. 3B). For many of these peptides it is unknown if they result in a T-cell response. However, closer inspection of these samples showed that 30 unique peptides were identified containing one or more known T-cell epitopes (Fig. 4; epitopes listed in Supplementary Table II). This finding illustrates that in conventionally produced gluten-free beers immunogenic gluten epitopes can occur, but evidently at safe levels below 10 mg/kg. In AN-PEP treated beers, not a single peptide sequence motif corresponding with even part of a known celiac disease relevant T-cell epitope was detected. Only a single peptide containing the R5 antibody epitope QQQFP was identified, having the sequence FISQSQQQFP that is present in γ-hordein. This peptide is indeed antigenic to the R5 antibody but probably not immunogenic, because it is not known as such and does not contain an internal proline (47,59). Overall, our results unequivocally demonstrated that beer stabilization treatment with AN-PEP also resulted in degradation of all known celiac disease relevant T-cell epitopes in beer to levels well below 20 mg/kg.

DISCUSSION

The enzymatic degradation of gluten from barley or wheat beers into smaller fragments could in principle be achieved by any peptidase. However, immunogenic gluten epitopes are characterized by the incidence of proline-rich stretches, and only a few endopeptidases are known that can efficiently cleave such peptides. AN-PEP is by far the best studied proline-specific endopeptidase that can specifically do this. In fact, AN-PEP is the only proline-specific endopeptidase that is industrially available and can cope with the acidic conditions of beer fermentation. The enzyme was developed to provide a convenient and commercially attractive means to prevent chill haze in beer. Chill haze develops as the result of an association of barley-derived proline-rich proteins (generally known as haze-sensitive proteins) with malt- and hops-derived polyphenols. Adding AN-PEP results in a selective but extensive breakdown of these sensitive proteins during the fermentation process, thus preventing the formation of large aggregates that become visible colloidal (chill) haze particles. We showed here that AN-PEP, used in levels commonly applied to prevent haze formation, also effectively degrades all known immunogenic gluten epitopes during beer production. Recently, the use of AN-PEP in other foodstuffs has been described (50,78).

Similar to the proline-rich haze-sensitive proteins, a fraction of the immunogenic proline-rich gluten epitopes can also be expected to survive a conventional brewing process. The actual presence and identification of such immunogenic gluten epitopes in beer by LC-MS/MS has been reported (11,65). Using our data-dependent discovery method on a high-end ultra-high performance LC-MS/MS system, we have been able to extend these existing data. Because beer contains many non-(chymo)trypsin produced fragments, a peptide identification procedure different from standard proteomics experiments had to be followed. First, we split the samples in three aliquots and treated one with trypsin, treated the second with chymotrypsin, and left the last untreated. Second, we determined the origin of the proteins present in the beers using trypsin-generated samples. Subsequently, all data were searched against a custom database containing all publically known protein sequences for *H. vulgare*, *S. cerevisiae*, and the sequences for trypsin, chymotrypsin, and AN-PEP with a "no enzyme" search setting. This allowed matching of the LC-MS/MS data to all possible peptides present regardless of any peptidase cleavage preference. The determination of a false discovery rate (FDR), which provides the quality control for the database search in regular proteomics experiments, is difficult for "no enzyme" database searches. FDR is typically determined in proteomics experiments by searching the data in a randomized or reversed database. This is appropriate for trypsin-treated samples, but in "no enzyme" searches the true sequences may also occur in randomized or reversed databases. The lack of an accurate FDR implies the need for extensive manual inspection of the raw data and database search results. We challenged this difficulty by manually inspecting the database search results using different filter settings. Finally, the precursor mass tolerance was set at <5 ppm to exclude most possibilities with divergent chemical compositions. The mass calibration of the Orbitrap LC-MS (Thermo Fisher Scientific) was carefully monitored to be within 2 ppm, and the peptide probability was set to >0.3. This setting was manually challenged by inspecting the spectral matches for spectra with peptide probability in this approximation range. To ensure that the matches thus identified were correct, a final manual inspection of the spectra matched to epitope-containing peptides was carried out.

The two conventionally processed beer samples (3 and 4) were gluten-free (<10 mg/kg) as judged by competitive R5 ELISA. However, LC-MS/MS peptide identifications on these two reference beers revealed the presence of many different known celiac-related gluten epitopes. Strikingly, such epitopes could not be detected in the corresponding AN-PEP treated beers (3* and 4*), thereby illustrating AN-PEP's efficacy in completely removing immunogenic gluten epitopes. These data also illustrate the sensitivity of the LC-MS/MS method. Another strength of the peptide identification strategy followed was that the identified peptide sequences could be linked to the proteins of origin and thereby to relevant biochemical and immunogenic knowledge. For example, the peptide QQQFP identified in AN-PEP treated beers could be traced back to the γ-hordein sequence FISQSQQQFP, confirming AN-PEP's proline-specific cleavage preference. Although peptide FISQSQQQFP is more than nine amino acids long, it is most likely not immunogenic because it does not contain an N-terminal or an internal proline, which is thought to be crucial for the transamidation reaction and therefore T-cell binding (47,59). In ELISA, however, the presence of this peptide could result in a false positive response because QQQFP is recognized by the R5 antibody.

Gluten epitope quantification in beer is subject to discussion, and both ELISA and MS approaches have been discussed extensively in combination with their potential differences in quantification limits (12,18,24,27,44,52,55,64,65,79). The Codex Alimentarius (10) requires that the gluten content of food prepared from cereals such as wheat and barley must be less than 20 mg/kg to be called gluten-free. This level is based on both technical grounds and safety grounds. Catassi et al. (9) showed that the lowest level to cause significant small bowel mucosa damage of celiac disease patients was 50 mg of daily gluten, if consumed for 3 months. Daily gluten intake of less than 10 mg was concluded to be unlikely to cause significant histological abnormalities (1). A 20 mg/kg level in all gluten-free foods and beverages was assumed to contribute to less than 15 mg/day of gluten. Of course, use of the correct antibody and most suitable control must be carefully considered. With these conditions in mind, the competitive R5 ELISA provides fast quantification of gluten in beer.

LC-MS/MS based quantification of gluten in beer, on the other hand, requires more effort. Selected reaction monitoring (SRM) or multiple reaction monitoring is proposed as a way around this. In this method, multiple peptides are monitored per protein after trypsin digestion to compare the abundance of the original protein in different samples. Multiple tryptic peptides cannot be selected for hordeins in beer in most cases owing to the high degree of endogenous hydrolysis and differential precipitation in the various steps of the brewing process. This is illustrated in Supplementary Figure 1, in which VFLQQQCSPVR was selected as the peptide for SRM quantification (65). The VFLQQQCSPVR sequence was identified in one peptide in the untreated fraction. Fragments of this peptide, however, were also identified in nine other peptides. These data demonstrate that the selected VFLQQQCSPVR peptide can resemble neither the molar amount of the original protein nor the molar abundance of gluten epitopes occurring in the protein. More alarmingly, this approach therefore introduces the chance of underestimating the gluten content in beer. The abundance of the epitope-containing peptides is of more importance than the original abundance of the intact hordeins. Quantification of epitope-containing peptides would require knowledge of all epitopes, and absolute LC-MS/MS based quantification requires labeled standards for all detectable epitope-containing peptides. Our discovery proteomics LC-MS/MS approach complements the ELISA approach by providing insight into the composition of gluten peptides present in beer and comparison with known gluten immunogenic sequences; however, accurate LC-MS/MS based quantification was not further explored.

The current experiments show that the industrial application of AN-PEP (Brewers Clarex) enables production of beer made from barley in which all known immunogenic gluten epitopes are undetectable.

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