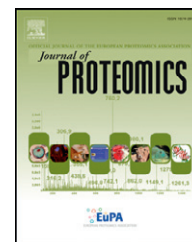


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Shotgun proteome analysis of beer and the immunogenic potential of beer polypeptides

Gianluca Picariello^{a,b,*}, Gianfranco Mamone^a, Chiara Nitride^{a,b}, Francesco Addeo^{a,b},
Alessandra Camarca^a, Immacolata Vocca^a, Carmen Gianfrani^{a,c}, Pasquale Ferranti^{a,b}

^aIstituto di Scienze dell'Alimentazione (ISA) — CNR, Via Roma 64, 83100 Avellino, Italy

^bDipartimento di Scienza degli Alimenti, University of Naples "Federico II," Parco Gussone, Portici (NA) 80055, Italy

^cEuropean Laboratory for the Investigation of Food Induced Diseases (ELFID), University of Naples "Federico II," Naples, Italy

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ABSTRACT

The majority of beer proteins originate from barley (*Hordeum vulgare*) which is used for brewing. Barley is known to contain celiacogenic gliadin-like prolamins (hordeins) along with other immunogenic proteins which endure malt proteases and the harsh conditions of brewing. In addition, a multitude of peptides that may retain or even amplify the immune-stimulating potential is released in beer because of proteolysis.

The comprehensive annotation of the beer proteome is challenged both by the high concentration range of the protein entities and by a severe degree of processing-induced modifications. Overcoming the pitfalls of the classical two-dimensional electrophoresis approach coupled to mass spectrometry (MS), the gel-free shotgun proteomic analysis expanded the current inventory of a popular Italian beer to 33 gene products, including traces of intact B- and D-hordeins and 10 proteins from *Saccharomyces* spp.

The high performance liquid chromatography–electrospray MS/MS peptidomic analysis of the low-molecular weight beer components disclosed a panel of hordein-derived peptides that encrypt gluten-like sequence motifs, potentially harmful to celiacs. The presence of antigliadin IgA-immunoresponsive prolamins was assayed by Western and dot blot using sera of N=4 celiac patients. Gliadin-reactive T-cell lines isolated from the intestine of N=5 celiacs activated an IFN- γ response when challenged with deamidated beer polypeptides.

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1. Introduction

Malt derived from germinated barley (*Hordeum vulgare* L.) and, to a much lower extent, from other cereal grains is the basic ingredient in brewing. Hordeins are the most abundant class of barley storage proteins, representing 40–50% of the total grain content [1]. Due to the partial sequence homology and to a certain degree of immune cross-reactivity with the wheat prolamins (gluten proteins), hordeins can trigger an immunoreaction in celiacs.

Hordeins are a complex polymorphic mixture of proteins coded by multi-gene families, which are usually classified into four groups named B-, C-, D- and γ -hordeins according to their electrophoretic mobility. B- (30–45 kDa) and C-hordeins (55–70 kDa) are the dominating sub-types accounting for 70–80% and 10–12%, respectively, while D- (105 kDa) and γ -hordeins (30–40 kDa) are minor components. B-hordeins are further categorized into B1, B2 and B3 sub-families [2].

More than 40 different proteases, in addition to amylases, are activated by malting [3]. The metabolic barley proteins

* Corresponding author at: Istituto di Scienze dell'Alimentazione (ISA) — CNR, Via Roma 64, 83100 Avellino, Italy. Tel.: +39 0825 299216; fax: +39 0825 781585.

E-mail address: picariello@isa.cnr.it (G. Picariello).

that are released in the water solution during mashing, along with a much lower amount of hordeins, which are 60% ethanol-soluble, are extensively hydrolyzed by the endogenous proteolytic enzymes [3]. Comparing the hordein extractability in reducing and non-reducing conditions from unmalted barley, barley and spent grain, it has been demonstrated that disulfide bonds engaged among B- and D-hordeins can undergo cleavage, thereby in part mobilizing hordeins that are extensively proteolyzed during malting. However, malting does not hydrolyze the entire hordein fraction. In contrast, mashing probably promotes the formation of novel disulfide links and induces the formation of aggregates that ultimately separate from wort [4]. Only minor amounts of intact proteins survive the further harsh brewing steps, which include wort boiling, fermentation and filtering. Thus, hordeins are progressively lost during brewing, considering that well-modified malts contain less than half the amount of the original barley. In addition, the level of anti-gliadin antibodies decreases at least three orders of magnitude in beer compared with raw malt [5].

It is well established that the beer proteome is dominated by Z-barley proteins and non-specific lipid transfer proteins (ns-LTPs). Z-barley protein, a ~43-kDa hydrophobic protein, is found in two isoforms: Z4 (80%) and Z7 (20%). Similarly two ns-LTPs occur in beer: ns-LTP1 of 9.7 kDa and ns-LTP2 of 7.0 kDa [6]. A number of minor protein components have been recently identified by exploiting upgraded proteomic strategies [7–9].

Nevertheless, despite the extensive investigation, the permanence in beer of intact hordeins is still under debate. Previous studies have well established that hordeins could play a critical role in inducing haze formation in beer [10]. Several studies conducted in the 1980s and 1990s, which were substantially based on the immunochemical detection with rather non-specific antibodies, appeared to support the occurrence of remarkable amounts of hordeins and their large-sized fragments in beer [11]. Therefore, beer consumption has been precautionarily banned from the diet of celiacs. More recent studies, mainly based on two-dimensional electrophoresis (2DE) coupled to mass spectrometry (MS), agree in excluding the permanence of major amounts of hordeins in beer [12,13], as only traces of γ -hordeins have been detected [14]. Large-sized fragments of D- and B-hordeins (17–20 kDa) were identified by SDS-PAGE and MS [15], while no evidence of their intact precursor has emerged. Exclusively minor levels of γ -hordeins were found in beer even when opportune strategies (ProteoMiner) were addressed to compress the dynamic range of the beer proteome [8]. The very recent off-gel prefractionation of four lager beers followed by narrow pI range 2DE analysis detected only traces of B-, γ - and fragments of D-hordeins [16].

Other previous studies demonstrated that not less than 60–80% of the proteinaceous beer fraction is represented by variably sized water-soluble proteolytic fragments [12,17,18]. Beer peptides have an intrinsic high heterogeneity degree that reflects the multiplicity of the barley storage proteins they arise from. The non-tryptic nature, the absolute lack of proteolytic specificity and the extensive non-enzymatic glycation severely challenge the characterization of the peptide fraction of beer, which awaits to be exhaustively characterized. The outcomes of our previous study [12], confirmed by a successive independent

investigation [9], have demonstrated that beer contains a variable amount of hordein-derived peptides and that some of these peptides encrypt the sequence motifs that are known to be involved in eliciting immunoreactions in celiacs. The amount of gluten-like sequences can significantly change as a function of the malt and of the processes that are adopted for brewing the uncountable beers currently on the market. However, R5 antibody-based competitive immunoassays [19] have demonstrated that in a high percentage of beers, the level of gluten-like epitopes is below 20 ppm, which corresponds to the precautionary threshold for gluten-free foods [20]. The technological use of prolyl endopeptidases or tannins during brewing has been proposed to deplete gluten-like epitopes [21]. In contrast, unsuspected sources of wheat gluten have also been identified in beers that are claimed to be all barley malt, thereby evoking the need for proper commercial labeling standards [7,9].

To explore the “deep” beer proteome and to definitely assess the occurrence of intact hordeins in beer, we utilized a gel-free shotgun proteomic strategy to analyze an Italian commercial beer, which has already been analyzed in the past by a 2DE-based approach [12]. The shotgun proteomic analysis, in general, overcomes many of the pitfalls of the classical 2DE analysis, enlarging the proteome coverage with special emphasis on the low-abundance components. In contrast to the recent works by Weber et al. and Colgrave et al. [7,9], to preserve the information about the persistence of intact proteins in beer, large-sized beer polypeptides were separated from peptides prior to performing trypsin digestion and microflow-high performance liquid chromatography (μ HPLC)/electrospray-tandem mass spectrometry (ESI-MS/MS) analysis. Beer proteins were analyzed either as a whole pool or after a fractionation step based on methanol solubility. With the aim of extending the characterization of the low molecular weight components, the <6 kDa peptide fraction was analyzed individually, both as undigested and after trypsin sub-digestion. Immunoreactive sequences were assayed by Western and dot blot, utilizing sera obtained from N=4 celiac individuals as a source of anti-gliadin IgA antibody. Finally, the capability of beer protein extracts to stimulate wheat gluten-specific T-cells was evaluated by determining the IFN- γ production in T-cell lines isolated from the intestine of N=5 celiac patients and highly reactive against gliadin.

2. Materials and methods

All reagents were of analytical or higher grade. Chemicals, acetonitrile (ACN), acetone, formic acid (FA), dithiothreitol (DTT) and iodoacetamide were from Sigma (Milan, Italy). Trifluoroacetic acid (TFA) was from Fluka (Milan, Italy). An Italian “single malt” (4.7% alcohol by volume) lager beer produced by Peroni (Roma, Italy; website: www.peroni.it) was obtained from the market. According to the producer's claims, the beer was brewed from two-row barley malt using blends of the varieties Alexis, Carina, Gitane, Cascade, Triumph and a low amount of unmalted corn (<15%). Protein content, determined by the Kjeldahl method ($6.25 \times N$), was 0.41 g/L and is consistent with the average values reported for other beers [16].

2.1. Protein and peptide purification

The degassed beer sample (aliquots of 1 mL) was chromatographed using an Econo-Pac® 10 DG column (Bio-Rad Laboratories, Hercules, CA, USA) by eluting with 50 mM Tris–HCl, 100 mM KCl, and 5 mM EDTA, at pH 7.0, to separate the protein from the low MW peptide fraction. Effluents were collected in fractions of 1 mL and monitored by the UV-absorbance at 280 nm (Ultrospec 2100 pro, Amersham Biosciences). The protein (MW > 6 kDa) and peptide (MW < 6 kDa) fractions were separately pooled. The peptide pool was further desalted using Sep-Pak® C₁₈ pre-packed cartridges (Waters, Milford, MA, USA) washed with aqueous 0.1% TFA (v/v) and eluted with 70% ACN (v/v)/0.1% TFA (v/v).

Aliquots of the protein extracts were further fractionated based on their solubility in methanol. In this case, the protein pool was diluted 5-fold with 0.1 M ammonium acetate in methanol at room temperature. Following incubation overnight at –20 °C, the methanol-insoluble fraction was pelleted by centrifugation at 4500 g for 15 min at 4 °C and washed three times with ammonium acetate in methanol and once with acetone [22]. Because the methanol-soluble pool generally contains “chloroform–methanol” or CM-like proteins, they are referred to as CM-soluble proteins. Proteins in the CM soluble proteins were precipitated with cold acetone, and the pellet was rinsed twice.

The entire protein extract, as well as the fractionated CM-soluble and CM-insoluble protein components, was dissolved at ~2 mg/mL in 0.3 M Tris–HCl, pH 8.0, containing 6 M guanidine hydrochloride and 10 mM DTT for 45 min at 55 °C. Cysteines were alkylated with a 5:1 molar excess of powder iodoacetamide with respect to the total –SH groups at room temperature for 30 min in the dark. The proteins were freed from low molecular weight compounds by filtration through Econo-Pac® 10 DG columns by eluting with 50 mM ammonium bicarbonate, pH 8.5. Protein concentrations were quantified by the Bradford assay. Aliquots of the low MW extract were reduced/alkylated according to the same protocol, desalted by Sep-Pak® columns and then trypsinized.

2.2. Trypsin hydrolysis

Protein and peptide samples were hydrolyzed with sequencing-grade modified trypsin (Promega, Madison, WI, USA) at an enzyme/substrate ratio of 1:100 w/w in 50 mM ammonium bicarbonate, pH 8.0, overnight at 37 °C. The samples were desalted using Sep-Pak® cartridges prior to μ HPLC–MS/MS analysis.

2.3. μ HPLC–MS/MS analysis

Both tryptic digests and non-proteolyzed beer peptides were separated by μ HPLC using an Integral 100Q HPLC system (PerSeptive Biosystems, Framingham, MA, USA). The flow rate was split from 200 μ L/min to 5 μ L/min using a flow splitter. Eluents: (A) 5% ACN in 0.08% FA and 0.01% TFA and (B) 95% ACN in 0.08% FA and 0.01% TFA. Peptides were separated on a capillary column, C₁₈ PepMap, 15 cm in length, 300 μ m ID, 300 Å (LC Packings), using the linear gradient 5–40% B over 60 min. For MS/MS analysis, a Q-Star Pulsar mass spectrometer

(Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ion source was used. The experiments were performed in the information-dependent acquisition (IDA) mode.

Precursor ions were selected for fragmentation using the following MS to MS/MS switch criteria: ions greater than m/z 400.0, charge states 1 to 4, intensity exceeds 15 counts, former target ions were excluded for 60 s and ion tolerance was 50.0 mmu. CID was used to fragment multiple charged ions, and nitrogen was used as the collision gas. Raw μ HPLC–MS/MS data sets were used to generate text files in mascot generic file format (.mgf). Peak lists were submitted to the Mascot (version 2.3) search engine (<http://www.matrixscience.com>) using the following criteria: databases, NCBI and/or Swiss-Prot; taxonomy, other green plants; type of search, MS/MS ion search; enzyme, trypsin; fixed modifications, carbamidomethyl; variable modifications, methionine oxidation and pyro-Glu formation through N-terminal loss of ammonia at Gln; mass values, monoisotopic; parent tolerance, 0.08 Da; ms/ms tolerance, 0.2 Da; number of maximum missed cleavages, 1.

Non-tryptic peptides were identified using the Batch-Tag Web tool of Protein Prospector (<http://prospector.ucsf.edu>), taxonomically restricting the search to *H. vulgare*, *Triticum* sp., *Zea mays* and *Saccharomyces cerevisiae*. The same search criteria used above were utilized in this case. Output peptide identifications were validated by manual inspection of MS/MS spectra. Peptide entries were considered identified when the measured molecular weight corresponded to the expected value and a sequence of at least three consecutive b- or y-ions occurred in the spectrum. For each of the protein/peptide fractions, μ HPLC–MS/MS analyses were run in triplicate, and only matches occurring in all the three analyses were included in the entry list.

2.4. Western blot and dot blot analysis

Whole beer protein extracts (10 μ g) were subjected to SDS-PAGE (12% gel) under reducing conditions on Bio-Rad (Hercules, CA, USA) mini gels (0.75 mm). Gliadins and hordeins, obtained by extracting soft wheat (cv. Mieti) and barley (cv. Giacinto) flour (100 mg) in 60% aqueous ethanol (0.5 mL, three extractions) after the removal of albumins and globulins by twice extraction with 1 mL of 0.4 mol/L NaCl, 0.067 mol/L Na₂HPO₄, pH 7.2 [23], were used as positive controls.

Gels were run at 220 V for 1 h and then electroblotted onto nitrocellulose membrane (Transblot nitrocellulose, Bio-Rad) at 120 V for 60 min. The membranes were blocked for 1 h using 5% non-fat milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T) for 1 h at room temperature (RT). Subsequently, the membranes were incubated overnight at 4 °C with human sera from celiacs or healthy individuals (negative controls) diluted 1/500 in TBS-T. After washing with TBS-T, monoclonal peroxidase-conjugated anti-human IgA antibody (Sigma) diluted in blocking solution (1/10,000) was applied to the membrane for 2 h at RT. The membrane was extensively rinsed with TBS-T (3 $\times$ 10 min) and finally with TBS (1 $\times$ 10 min) before development. Chemiluminescence reagents (ECL Plus WB reagent, GE Healthcare) and X-ray film (Kodak, Chalon-sur-Saône, France) were used to visualize the immunoreactive protein bands at various exposure times.

For dot blot assay, an aliquot of the protein/peptide fractions ($\sim 0.1 \mu\text{g}$) was spotted onto 0.45- μm Transblot nitrocellulose membranes, air dried and developed using the same protocol as the Western blot assay. The whole gliadin and hordein extracts were used as the positive control, while the ethanol extract from maize flour was used as the negative control. Beer proteins/peptides were also assayed after tissue transglutaminase (tTGase)-mediated deamidation using an aliquot of tTGase ($\sim 0.01 \mu\text{g}$) as the control.

2.5. Sequential peptic–tryptic digestion of beer polypeptides

To assay the T-cell stimulatory activity of the polypeptide fraction of beer, an aliquot of degassed sample (10 mL) was lyophilized after protein quantification by Bradford assay and subjected to sequential peptic–tryptic (PT) digestion. Briefly, lyophilized beer was re-suspended in 3 mL of 5% formic acid and incubated for 1 h at 37 °C with pepsin (enzyme-to-substrate ratio $\sim 1/50$, w/w). The reaction was stopped by freeze-drying, and tryptic digestion was performed in 50 mM ammonium bicarbonate, pH 8.0, for 4 h at 37 °C at an enzyme-to-substrate ratio of $\sim 1/50$, w/w. The peptides were lyophilized and reconstituted with water three times and purified by C₁₈ Sep-Pak® cartridges, as described.

2.6. Deamidation of gliadin-like beer peptides

Beer PT-digests were re-suspended in 50 mM Tris–HCl buffer (pH 6.8) containing 5 mM CaCl₂, 10 mM NaCl and 10 mM DTT and incubated at 37 °C for 4 h with tissue-transglutaminase (tTGase, Sigma) at a 1/10 enzyme/substrate ratio. The reaction was arrested by incorporating up to 10 mM EDTA as the final concentration.

2.7. Generation of gliadin-specific T-cell lines and T-cell assays

Gliadin-specific intestinal T-cell lines (iTCLs) were generated from duodenal biopsies of 5 HLA DQ2-positive CD patients (2 untreated and 3 treated, mean age 24 years, range 5–36 years), as described previously [24]. Mucosal cells were stimulated for 2–3 cycles with irradiated autologous peripheral blood mononuclear cells and deamidated PT-gliadin extracted from the hexaploid wheat variety Sagittario. Flow cytometry analysis revealed that all iTCLs contained more than 90% of CD3/CD4 positive cells. The immune response to both native and deamidated beer protein extracts was assayed by the detection of IFN- γ production by ELISA, as described previously [24]. Briefly, T-cells (3×10^4) were incubated with autologous B-LCLs (1×10^5) in the presence or absence of PT-gliadin-TG (50 $\mu\text{g}/\text{mL}$) or PT-beer $\pm$ TG (100 $\mu\text{g}/\text{mL}$) in 200 μL of complete medium (X-Vivo plus 5% human serum, Lonza-BioWhittaker, Verviers, Belgium) in U-bottom 96-well plates. Cell supernatants were collected after 48 h for determination of IFN- γ . Each antigen preparation was assayed in duplicate and in at least three independent experiments. As a criterion for a positive response, a twofold response compared with the background value (IFN- γ production to medium alone) was arbitrarily chosen, in agreement with previous reports [24].

3. Results and discussion

3.1. Shotgun proteomic analysis of beer

Previous proteomic investigations support a strict consistence of the beer proteome regardless of the brand under a qualitative standpoint, while detectable differences appear confined to the relative quantitative balance among protein components. Thus, a popular Italian commercial beer, which we previously analyzed [12], was selected as a representative sample for the shotgun proteomic analysis. Protein and peptide fractions were analyzed according to the workflow shown in Fig. 1.

The preliminary size exclusion chromatography (SEC) step was a gross separation of the large-sized beer polypeptides (here referred to as the “protein” fraction) from the <6 kDa peptide fraction. This step allowed the preservation of the persistent intact proteins in beer and/or their large-sized fragments. Similar to previous findings by H. Konečná et al. [16], SEC enabled a much higher protein recovery with respect to the precipitation procedures. Protein mixtures were reduced/alkylated, and the tryptic digests were analyzed by $\mu\text{HPLC}/\text{ESI-MS}/\text{MS}$. Shotgun proteomic analyses were performed on the protein fraction either as a whole pool or following a fractionation step based on methanol solubility (CM-soluble and CM-insoluble fraction). This last pre-fractionation was introduced to further partition the protein suite into subsets to reduce the impact of the large protein dynamic range on the analysis.

The list of significant protein hits from the Mascot search is shown in Table 1. Overall, 33 different gene products were identified, which included 20 from *H. vulgare*. According to the emPAI label-free quantitative estimation [25], ns-LTPs were identified as the most abundant proteins in beer, although a

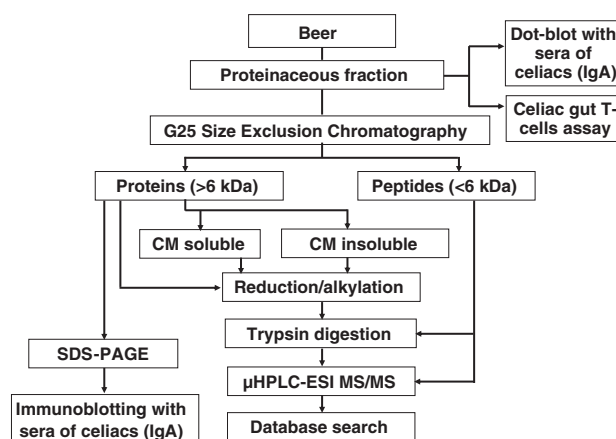


Fig. 1 – Schematic of the workflow for the shotgun proteomic and peptidomic analyses of beer. Proteins (>6 kDa) and peptides (<6 kDa) were previously fractionated by SEC to preserve the information about the persistence of intact proteins and/or their large-sized fragments in beer. To reduce the impact of the dynamic range, proteins were further fractionated according to their methanol solubility prior to shotgun analysis.

portion of these proteins was lost in the <6 kDa fraction, as it was ascertained with the μ HPLC/ESI-MS/MS analysis of the low MW fraction. Z-barley proteins scored among the dominant components, although not the most abundant, differently from what was expected according to the 2DE evidence. Thus, in this case, the emPAI quantification has to be considered only as a rough estimation of the relative protein amounts.

Ns-LTPs and Z-barley proteins are heat-stable, and they endure, at least in part, the action of digestive enzymes due to their specific roles as protease inhibitors. The immunological active domains of these proteins that survive gastrointestinal digestion can elicit IgE-mediated sensitization in predisposed individuals through mechanisms that are not related to celiac disease [26]. As a general trend, the harsh processes of brewing operate a process of selection, enriching a restricted panel of barley endosperm components categorized among the barley seed pathogenesis-related proteins [27], including among others primarily inhibitors of proteases and/or amylases [28]. The avenin-like protein A, identified by homology

from *Triticum aestivum*, significantly contributed to the protein fractions. Although they have been discovered in beer only recently, it is now established that avenin-like protein A and related isoforms, which share sequence homology with γ -hordeins, are to be counted among the major beer proteins [7,9,12]. Hiemori et al. described a novel 18-kDa barley-derived allergen in beer [29]. Although the protein remained uncharacterized, the 2DE map coordinates point to the likelihood of this protein being the avenin-like protein A.

B1-, B3-, D- and γ -hordeins were identified in the protein fraction of beer, although in relatively low amounts. It is important to acknowledge that the shotgun proteomic analysis proved to be more effective than the classic 2DE-based approach in identifying the low-abundance components, such as hordeins. Similarly, proteins such as barwin, which is a highly alkaline protein, normally escape the 2DE detection. Barwin was detected in beer by a mono-dimensional electrophoresis and MS-based approach [15] and later confirmed after ProteoMiner enrichment [8]. Recently, barwin peptides

Table 1 – Identification of beer proteins by the shotgun proteomic approach. For a label-free rough quantitative estimation, emPAI values [25] have been evaluated by searching the file of the whole protein pool or, alternatively, a unified mgf file of both methanol insoluble and soluble fractions.

Identification	Organism ^a	Uniprot accession number	MW	pI	Score	Peptide matches (unique)	emPAI	Protein fraction ^b
Non-specific lipid transfer protein 1	H	P07597	9.7	8.19	344	23 (8)	2.52	W, I, S
Z4-type serpin	H	P06293	43.3	5.72	236	32 (12)	0.58	W, I
Avenin-like protein A3	T	P0CZ08	16.3	8.24	200	5 (5)	0.52	W, I, S
D-hordein	H	Q40054	75.1	8.00	159	11 (6)	0.08	W, I, S
Non-specific lipid transfer protein 2	H	P20145	7.0	6.98	146	9 (4)	1.67	W, I, S
α -amylase/trypsin inhibitor CMd	H	P11643	16.1	5.24	125	8 (4)	0.54	W, I, S
α -amylase inhibitor BDAI-1	H	P13691	16.4	5.36	109	11 (6)	0.38	W, I, S
Trypsin inhibitor CMe	H	P01086	16.3	7.51	105	4 (2)	0.40	W
Enolase 1	S	P00924	46.8	5.67	95	7 (6)	0.20	W, I
α -amylase/trypsin inhibitor CMb	H	P32936	16.5	5.77	92	12 (8)	0.89	W, I, S
γ -hordein 3	H	P80198	33.2	6.70	90	7 (3)	0.29	W
Trypsin/amylase inhibitor pUP13	H	225102 ^c	14.7	5.35	80	9 (3)	0.70	W, I, S
B3-hordein	H	P06471	30.2	7.74	79	2 (1)	0.10	I, S
Z7-tye Serpin	H	Q43492	42.8	5.45	74	8 (5)	0.22	W, I
γ -hordein 1	H	P17990	32.7	8.12	71	3 (2)	0.08	S
Cell wall protein PIR 5	S	A6ZQH2	23.5	8.68	62	5 (3)	0.10	I
Late embryogenesis abundant protein EMB564	M	P46917	9.7	6.61	59	1 (1)	0.31	W, I
Hordoindoline A	H	Q9M4E3	13.2	8.60	58	3 (2)	0.28	W
Barwin	H	P28814	13.7	7.76	54	1 (1)	0.21	I
Grain softness protein	H	Q5ITH7	18.2	4.97	52	1 (1)	0.16	I
Predicted 76.9 kDa protein	H	F2EBM4	76.9	5.40	49	1 (1)	0.04	I, S
α -amylase/trypsin inhibitor CMa	H	P28041	15.5	5.87	48	5 (4)	0.40	W, I
Cell wall protein AWA1	S	Q8TGE1	162.0	4.15	47	2 (2)	0.03	I
Triosephosphate isomerase	S	P00942	26.8	5.74	45	3 (3)	0.23	I
Glucan 1,3- β -glucosidase	S	P23776	46.9	4.45	44	1 (1)	0.06	I
Protein SIM1	S	P40472	46.3	4.40	44	1 (1)	0.06	I
O3625p protein	S	C8ZGT4	49.4	9.21	41	1 (1)	0.07	I
Avenin-like protein A4	T	D2KFH1	17.0	7.98	41	1 (1)	0.15	I, S
Increasing suppression factor 1	S	P32488	37.1	8.99	38	1 (1)	0.08	I
Protein PRY1	S	P47032	28.7	4.31	38	1 (1)	0.09	I
B1-hordein	H	P06470	31.5	6.54	37	1 (1)	0.14	W
NBS-LRR type resistance protein	H	Q48972	54.7	7.70	33	1 (1)	0.05	I
Alr2p transporter	S	E7Q3C5	92.3	5.73	34	7 (1)	0.03	I

^a H=*Hordeum vulgare*; T=*Triticum aestivum*; S=*Saccharomyces cerevisiae*; M=*Zea mays*.

^b W=whole protein fraction; I=methanol insoluble; S=methanol soluble.

^c NCBI accession.

were found to be covalently cross-linked to the Z4-protein in boiling wort, confirming the occurrence of a certain degree of sulfhydryl exchange induced by the thermal processes of brewing [30]. In contrast, unhydrolyzed C-hordeins did not occur in a detectable amount, as previously demonstrated [9]. C-hordeins consist of multiple repeats of the PQQPFPQQ octapeptide that render them highly water-insoluble.

Only one of the identified proteins arose from *Z. mays*. Consistent with previous studies that indicated the need for enrichment procedures to identify the scarcely represented proteins released by *S. cerevisiae* [8,16], the pre-fractionation step based on the methanol solubility was decisive for the identification of 9 out of 10 *S. cerevisiae*-derived proteins.

3.2. Peptides of beer

The low MW peptide fraction of beer is extremely complex because of many concurring factors including the extreme heterogeneity of the parent proteins and the extensive non-enzymatic glycation (Maillard reaction) induced by thermal treatments [31,32]. The non-tryptic nature of the peptides and the absolute lack of cleavage specificity during protein hydrolysis further complicate a comprehensive characterization of this fraction.

The MALDI-TOF MS analysis of beer peptides confirmed the complexity of the mixture that contains components spread over the entire 1–10 kDa MW range, as previously shown [12,33]. The 6-kDa limit of exclusion of the Econo-Pac 10 DG columns was only indicative, considering that the entire 9.6-kDa ns-LTP1, also in variously glycosylated forms, was clearly detected by MALDI-TOF MS in the peptide fraction [33]. Because larger peptides were detrimental to the IDA ESI-MS/MS analysis, a preliminary peptide fractionation was performed using a 3-kDa cut-off membrane.

To enlarge the peptidomic inventory of beer, both the <3 kDa and >3 kDa fractions were analyzed by μ HPLC-ESI-MS/MS either in the absence of further enzymatic digestion or after trypsinolysis following the reduction/carboamidomethylation of cysteines. The shotgun analysis of the >3 kDa fraction only allowed the retrieval of the previously identified ns-LTPs and α -amylase/trypsin inhibitors. Table 2 reports the comprehensive list of peptide fragments identified in beer. The repertoire of beer peptides is far from exhaustive elucidation due to its complexity. Furthermore, the peptide components are expected to be significantly different between the commercial beer brands depending on the raw material and brewing technology. The identified peptides arise primarily from the proteins already identified in the high-MW fraction. Current data confirm that beer contains a large number of partially degraded hordein fragments. The hordein-derived peptides can encrypt several motifs (QQPY, QPYP, PQQPY, and QQPYP) associated with the induction of celiac disease [34–36]. The PQQPY motif contained in B-hordein-derived fragments is also contained in the toxic peptide 31–43 of α -gliadin. The PQQP sequence, included in both B- and C-hordein fragments is a repeated motif within the 26-mer toxic peptides identified by Shan et al. [37]. More precisely, the PQQPF of the C-hordein fragment has been regarded as one of the main gliadin toxic motifs, which are additionally resistant to digestion by gastric and pancreatic enzymes [38]. Furthermore, the encrypted QQPFP is the

pentapeptide recognized by the R5 monoclonal antibody, now largely used for detecting gluten in foods.

The 7-amino acid sequence PQQYPYQ (see Table 2) contains two overlapping harmful 4-amino acid motifs. This sequence is entirely homologous to the repeated domain of several wheat α/β -gliadins. QQPFP of a B-hordein fragment is common to the toxic 33-mer 56–88 of α 2-gliadin [39]. The extended QQPFP motif found in beer extracts is common to the 25-mer gastrointestinal resistant peptide, which is thought to be a potential trigger of celiac disease [40]. Interestingly, hordein fragments were only identified in the <3 kDa peptide fraction, suggesting that hordeins in beer might be the most extensively degraded.

3.3. Immunochemical detection of gluten-like responsive polypeptides

Both the protein and peptide fractions of beer were assayed for their antigenic activity against IgA-antibodies in sera from four celiac patients. The dot blot analysis revealed that either beer proteins or peptides were IgA immune-responsive for 4/4 sera (not shown). As expected, the 60% ethanol extracts from wheat and barley control flour were clearly IgA reactive, while no detectable reactivity was observed for maize prolamins (zeins) used as the negative control.

The tTGase-induced deamidation of prolamins has been demonstrated to strongly increase their affinity for HLA-DQ2 or -DQ8 molecules [41,42]. The detection of gliadin antibodies directed against the native disease-provoking cereal proteins suffer from poor specificity and sensitivity. In contrast, antibodies that recognize deamidated gliadin epitopes appear to provide more reliable diagnostic indications about active celiac disease [43].

Some of the identified sequences contained the typical “consensus” motifs susceptible of tTGase induced deamidation [44]. Thus, it can be arguably hypothesized that the affinity of beer-derived peptides for anti-gliadin IgA of celiacs is enhanced by tTGase. Nevertheless, we did not observe substantial discrepancies in the recognition patterns of beer proteins/peptides before and after deamidation. To this purpose, it must be considered that some Gln-rich hordein-derived peptides of beer were already characterized by MS/MS as non-specifically deamidated due to brewing-induced modifications (Table 2). tTGase, used as a control in dot blot experiments, showed only a barely detectable reactivity, demonstrating that the IgA antibodies have a specific affinity for the peptide sequences of beer. Serum from control non-celiac children did not exhibit detectable IgA reactivity against beer proteins/peptides and their deamidated counterparts.

Altogether these results demonstrate that the humoral immune response of celiacs against prolamins has to be considered active against gluten-like epitopes of beer.

To identify the specific immune-responsive protein component(s) we performed Western immunoblot analysis, evaluating the IgA reactivity. In this case, whole gliadins and hordeins were used as the positive controls and zeins served as the negative controls (Fig. 2). For all of the four sera, beer displayed a single immunoreactive band with a MW of approximately 75 kDa, compatible with the D-hordein also identified by μ HPLC-ESI-MS/MS. No additional IgA reactive

Table 2 – μ HPLC-ESI-MS/MS-based identification of low molecular weight peptides of beer. Except where indicated, the peptides were identified by the Batch-Tag Web tool of Protein Prospector. The sequences potentially implicated in eliciting an immunological response in celiacs are highlighted in bold.

MH ⁺	m/z	z	Delta	Sequence	Modification(s)	Score	Note
nsLTP 1 (<i>H. vulgare</i>) P07597							
479.2506	479.2538	2	0.003	VPYT		M	
659.3252	659.3261	1	9.0e–4	ISPDID		M	
894.4798	447.7458	2	0.004	GIHNLNLN		M	
1237.6290	619.3251	2	0.013	GIHNLNLNNAAS		M	
1662.9645	831.9859	2	0.036	GIHNLNLNNAASIPSK		40.2	
2122.9902	1062.0398	2	0.041	CNVNVPTYTISPDIDCSRI		26.9	RCM
2009.9061	1005.4937	2	0.037	CNVNVPTYTISPDIDCSR		31.1	RCM
nsLTP 2 (<i>H. vulgare</i>) P20145							
1495.7038	748.3888	2	0.034	DTLNLCGIPVPHC		31.1	RCM
α-amylase/trypsin inhibitor Cmd (<i>H. vulgare</i>) P11643							
761.4198	761.4248	1	0.005	FPTNLLG		M	
1876.0228	626.0268	3	0.015	LLVAPGQCNLATIHNV		33.5	RCM
1762.9387	588.3351	3	0.018	LVAPGQCNLATIHNV		18.6	RCM
2286.0760	762.7199	3	0.023	AAAATDCSPGVAFPTNLLGHCR		21.4	RCM
2144.0018	715.3589	3	0.020	AATDCSPGVAFPTNLLGHCR		32.9	RCM
1813.8795	907.4689	2	0.026	DYVLQQTCAVFTPGSK		29.0	RCM
α-amylase inhibitor BDAI-1 (<i>H. vulgare</i>) P13691							
2232.2175	744.7604	3	0.017	LLVAGVPALCNVPIPEAAGTR		36.6	RCM
α-Amylase/trypsin inhibitor ClCH₃/MeOH-soluble protein (CMe) (<i>H. vulgare</i>) P01086							
1032.5591	516.7843	2	8.5e–4	DALPHNPLR		M	
α-amylase/trypsin inhibitor CMa (<i>H. vulgare</i>) P28041							
957.4710	957.4798	1	0.009	MGLPSNPLE		M	
B3-hordein (<i>H. vulgare</i>) P06471							
1249.7639	625.3856	2	0.017	VQIPFVHPSIL		31.1	
1377.8601	689.4337	2	0.036	VQIPFVHPSILQ		35.4	
1604.9843	802.9958	2	0.034	VQVQIPFVHPSILQ		32.5	
921.4833	461.2453	2	0.018	QFPFPQQPP ^a	Gln→pyro-Glu1	23.4	
1049.5059	525.2566	2	4.1e–4	QFPFPQQPP ^a	Gln→pyro-Glu1	19.4	
1022.6211	511.8142	2	0.0089	IPFVHPSIL		32.6	
1144.6775	572.8424	2	0.028	SIVLQEQLSQ		19.4	
1136.7147	568.8610	2	0.034	VQIPFVHPSI		19.7	
1605.8431	803.4252	2	0.037	QFPFSPQFPFPQQPP	Gln→pyro-Glu1	23.1	
1612.8727	806.9400	2	0.030	QFPFPQQPIQQP	Gln→pyro-Glu1	23.1	
1177.6071	589.3072	2	–0.0070	PQQIIPQQPQ	Deamid. at 10 or 7	19.6	
830.4883	415.7478	2	0.0076	AIDTRVG		25.3	
731.4745	366.2409	2	0.035	AIDTRVG		17.4	
1050.0971	525.2765	2	0.020	QFPFPQQP	Gln→pyro-Glu1	19.8	
1628.8599	814.9336	2	0.027	QPIPPQQPYPQQP	Gln→pyro-Glu1	21.6	
1153.5489	577.2781	2	–0.0074	SQQFPFPQQP		18.6	
B1-hordein (<i>H. vulgare</i>) P06470							
1049.5661	525.2867	2	0.012	QKFPFPQQP	Gln→pyro-Glu1	20.9	
1080.5495	540.7784	2	0.0011	QKFPFPQQP		20.7	
1163.5879	582.2976	2	0.0017	PQQFPFPQQP	Gln→pyro-Glu1	21.0	
1290.6515	645.8294	2	0.020	QFPFPQQPQP ^b	Gln→pyro-Glu1	16.8	
B-hordein (<i>H. brevisubulatum</i> subsp. <i>turkestanicum</i>) Q670S1							
857.4152	857.4152			PQQFPYPQ		M	
Beta-amylase (<i>H. vulgare</i>) P16098							
1496.8213	748.9143	2	0.032	PYVDPMAPLPRSGP		34.5	
1151.6197	576.3135	2	0.016	PMAPLPRSGPE		17.8	

Table 2 (continued)

MH ⁺	m/z	z	Delta	Sequence	Modification(s)	Score	Note
<i>γ</i> 3-hordein (<i>H. vulgare</i>) P80198							
603.3507	603.3549	1	0.004	FVLPQ		M	
773.4773	773.4923	1	0.015	SLVIQTL		M	
1874.8392	937.9378	2	0.015	PMLCNVHVPPYCSPPG		24.4	RCM
1926.0625	963.5349	2	0.038	QQPGQAFVLPQQQAQFK	Gln→pyro-Glu1	21.5	
1028.6955	514.8514	2	0.030	VVGSLVIQTL		29.5	
1201.6023	601.3048	2	0.0012	QWQPLPQQPP	Gln→pyro-Glu1	19.4	
1290.7193	645.8633	2	0.043	PQQQPLPQQP	Deamid. 4 or 7 and 9	17.2	
1080.5495	540.7784	2	0.019	QQQPPQQP	Gln→pyro-Glu1	18.0	
Predicted protein (<i>H. vulgare</i>) potassium ion transmembrane transporter activity F2CXQ9							
1302.6694	651.8768	2	0.076	SLDNELSFHKK		M	
Predicted avenin like protein (<i>H. vulgare</i>) F2EGD5							
1729.9580	865.4829	2	0.044	SFGQPQQQVPVEVMR		20.2	
C-hordein (fragment) (<i>H. vulgare</i>) P17991							
1652.8289	826.9181	2	0.0055	QQPQQPFPLQPHQP	Gln→pyro-Glu1	26.9	
1177.6243	589.3158	2	0.030	QPQQPFPPQQP ^c	Gln→pyro-Glu1	19.8	
Hordoinoline-A (<i>H. vulgare</i>) Q9M4E3							
1143.5879	572.2976	2	0.015	DLGGFFGFQR		24.1	

M=targeted manual assignment (according to ref. [12]).
RCM=identified only by analyzing tryptic digests after Cys-reduction/carboamidomethylation.
^a Peptide common to B3- and B1-hordeins.
^b Peptide also occurring in C-hordein ([P17992](#)).
^c Peptide also occurring in *γ*-hordein-1 ([P17990](#)).

bands were observed in beer. The immuno-reactive band was not detected by blue silver-stained SDS-PAGE; therefore, it was not possible to proceed further for a definitive characterization (i.e., peptide mass fingerprinting). The control serum of a healthy individual did not recognize any of the protein bands (not shown). While shotgun proteomics enabled the identification of large polypeptides belonging to the B-, *γ*- and D-hordein subtypes, no immune-responsive bands ascribable to them were detected.

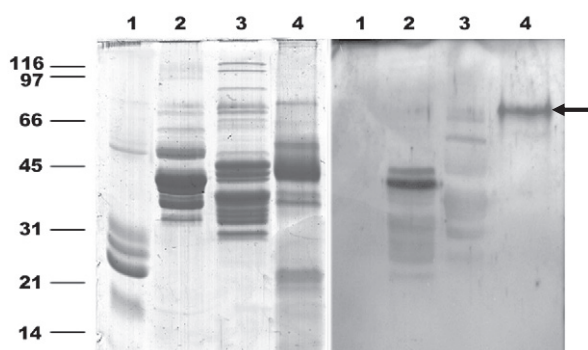


Fig. 2 – SDS-PAGE and Western immunoblotting of beer proteins (Lane 4). Proteins were immunostained with the sera of celiac patients (N=4) as the source of anti-gliadin IgA. Gliadins (Lane 2) and hordeins (Lane 3) were used as the positive controls. Zeins (Lane 1) were used as the negative control. In beer, only a ~75-kDa D-hordein band, indicated with an arrow, was immunoreactive.

3.4. T-cell stimulatory activity of the polypeptide fractions of beer

The immune stimulatory properties of the whole beer protein/peptide extracts were further investigated by challenging gliadin-reactive T-cell lines isolated from small intestinal mucosa of 5 HLA-DQ2+ celiac individuals.

PT-digests of the entire polypeptide fraction of beer were assayed both before and after tTGase-mediated deamidation, a process that highly increases the capability of gluten peptides to stimulate celiac T-cells [45,46]. Previous reports show that gluten contains several immunogenic sequences, and importantly, many of these epitopes are highly homologous and cross reactive with sequences present in barley and rye [36]. Therefore, we used a sensitive bioassay utilizing polyclonal CD4+ T cells challenged with deamidated whole gliadin and reacted against a heterogeneous panel of peptides mapping all three gliadin main families (alpha, gamma and omega), as shown in Table 3. As shown in Fig. 3, the beer protein extracts induced significant IFN- γ production by T-cells from celiac intestinal mucosa when compared to unstimulated cells (medium alone). This induction occurred in all of the investigated iTCLs. Additionally, the stimulatory properties were comparable to those induced by hexaploid gliadin in 3 out of 5 iTCLs (CD061204, CD410051, and CD131107) at a concentration of 100 μ g/mL of beer extracts. By contrast, as expected, the non-deamidated beer proteins were either less active or showed no activity with the only exception of TCL CD230204, which also reacted to non-deamidated wheat gliadin (data not shown).

Table 3 – DQ2-restricted gluten peptides recognized by celiac mucosa T-cell lines.

Patient	α -Gliadin	γ -Gliadin	ω -Gliadin
CD230204	33mer (DQ2.5-glia- α 1a/b, DQ2.5-glia- α 2) ^a	DQ2- γ -V (DQ2.5-glia- γ 5, -glia- γ 3)	
CD061204		14-mer-1 (DQ2.5-glia- γ 2)	
CD041051		DQ2- γ -I (DQ2.5-glia- γ 1) 14-mer-2 (DQ2.5-glut1)	DQ2- ω -I (DQ2.5-glia- ω 1, -glia- ω 2)
CD131107	33mer (DQ2.5-glia- α 1a/b, -glia- α 2)		DQ2- ω -I (DQ2.5-glia- ω 1, -glia- ω 2)
CD280900		DQ2- γ -I, -II, -III (DQ2.5-glia- γ 1, -glia- γ 2, -glia- γ 3)	

^a New peptide nomenclature is indicated within parentheses [49].

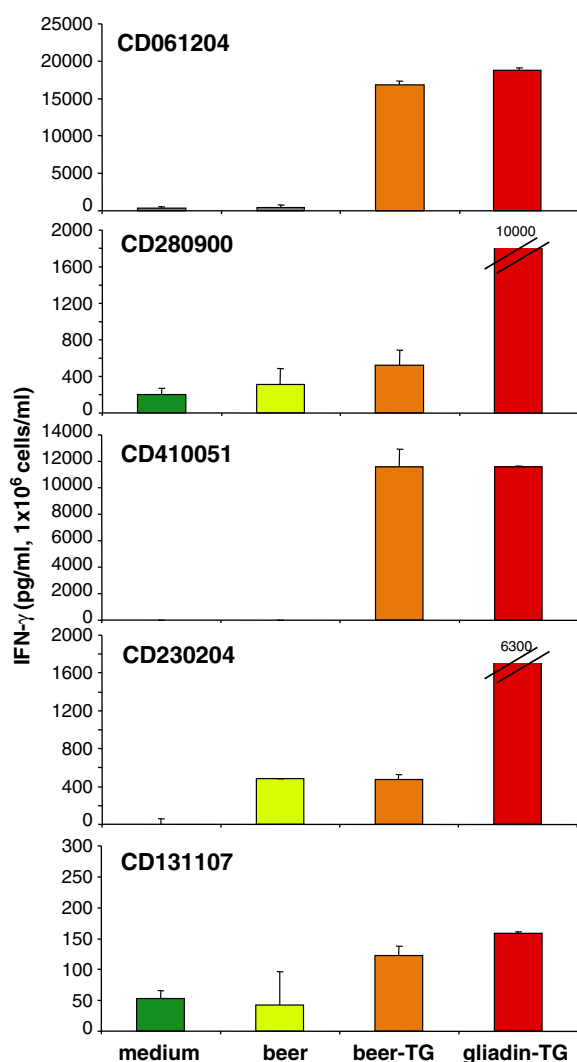


Fig. 3 – Stimulatory properties of beer peptide extract were assayed by detecting IFN- γ production of T-cell lines obtained from the intestinal mucosa of 5 celiac patients. iTCLs were raised against deamidated PT-gliadin from hexaploid wheat (gliadin-TG) and tested for cross-reactivity against PT of beer polypeptides (100 μ g/mL) before (beer) and after tTGase-mediated deamidation (beer-TG). IFN- γ levels detected in cell supernatants by standard sandwich ELISA are shown as the mean $\pm$ SD.

The capability of beer protein extract to stimulate T-cells representing celiac disease can be explained by a marked sequence homology between hordein- and gliadin-derived peptides. The reaction results in an extensive immune cross-reactivity between wheat and barley, which is well documented by previous studies [36,47,48]. In particular, the immunodominant DQ2.5-glia- ω 1 and - ω 2 peptides are present in wheat ω -gliadins and in barley hordeins. Furthermore, hordeins contain at least two fragments that are identical to the γ -gliadin-derived epitopes DQ2.5 glia- γ 5 and DQ2.5 glia- γ 3 [36,47] and additional sequences highly homologous to other γ -gliadin epitopes [47,49]. In this regard, the well-documented heterogeneous profile of peptide reactivity by celiac iTCLs could also explain the large variability of IFN- γ magnitude obtained in response to either beer proteins or hexaploid gliadin, especially after tTGase deamidation [36,48,49].

In conclusion, our results unequivocally demonstrate that beer contains celiacogenic sequences susceptible to tTGase and recognized by polyclonal T-cell lines, providing a direct evidence of the persistence in beer of antigenic sequences that need to be accurately type-by-type quantified for consumption by celiacs.

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