



# Immunoanalytic investigation of grain proteins antigenic for celiac disease patients in an einkorn collection

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## ABSTRACT

Our study focuses on the complex characterization of a wild and cultivated einkorn collection of the Cereal Gene Bank of Agriculture Research Institute in Hungary, using proteomics, immune analytics and bioinformatics analyses. In a serological ELISA pre-screen of 208 different *Triticum monococcum* L. ssp. *monococcum* and *Triticum monococcum* L. ssp. *aegilopoides* genotypes with celiac disease samples high diversity was observed in the immune response. Based on the immune analytic results, four genotypes with significantly reduced immune reactivity were selected for detailed proteomics characterization. Our results confirm the benefits of high-throughput/large-scale pre-screening and the use of a complex examination platform to get relevant information about the genetic diversity of celiac disease-relevant proteins in the analyzed einkorn genotypes. These genotypes cannot be incorporated into the daily diet of celiac patients; however, they may represent candidates – especially in combination with enzymatic treatments - to improve the lifestyle of individuals suffering from other clinical conditions like non-celiac wheat sensitivity.

## 1. Introduction

Einkorn (*Triticum monococcum* L. ssp. *monococcum*, A<sup>3</sup>) is one of the earliest cultivated forms of wheat, alongside emmer wheat (*T. turgidum* ssp. *dicoccum*, BA<sup>4</sup>). Einkorn can refer either to the wild diploid species of wheat, *T. monococcum* L. ssp. *aegilopoides*, or to the domesticated form, *Triticum monococcum* L. ssp. *monococcum*. Einkorn is a diploid species of hulled wheat, with tough glumes ('husks') that tightly enclose the grains. The cultivated form is similar to the wild relative, except that the ear stays intact when ripe and the seeds are larger. Einkorn has higher macro- and micronutrient content (minerals, carotenoids), lower carbohydrate and nearly double protein content than bread wheat and is

easily digestible (Abdel-Aal et al., 1995; Shewry, 2018; Geisslitz et al., 2019). Despite the 30–42% lower grain yields of these ancient wheats and their inferior baking properties compared to common wheat (Longin et al., 2015), in the last decades consumers associate the products made of these wheats with improved health benefits (Shewry 2018, Geisslitz et al., 2019). In einkorn, similar to related species such as wheat, barley or rye the major storage proteins belong to the prolamin proteins and account for about 60–80% of total grain proteins depending on genotypes (Bancel et al., 2019). They represent an important source of plant-based proteins in human nutrition, however many of the prolamin type grain proteins are considered as trigger molecules in the development of celiac disease or contribute to the development of

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symptoms in food allergy, baker's asthma, non-celiac wheat sensitivity or wheat-dependent exercise-induced anaphylaxis (Sollid et al., 2020). In addition to their storage protein functions, multiple members of the prolamin gene family are involved in stress defense-related mechanisms (Juhász et al., 2018).

The genetic variability of einkorn is significantly larger than in bread wheat (Desheva and Kyosev, 2016), which is also reflected by its more diverse storage protein composition and a lower epitope density in the gluten protein sequences (Juhász et al., 2018; Malalgoda et al., 2019).

Einkorn is among the well-studied diploid wheats with low immune response in patients with celiac disease compared to other wheat species, however, it is not considered safe for celiac patients (Picascia et al., 2020).

In the last two decades, there is a significant increase in the number of individuals who follow a gluten-free diet, either due to medical conditions developed after gluten consumption or as a personal choice. This trend draws increased attention to find wheat sources with decreased gluten content. Although there exist natural and genetically modified mutants lacking either one or multiple storage protein loci (Pistón et al., 2011; Altenbach et al., 2019) or cultivars with modified gluten protein expression, it is rather challenging to develop wheat cultivars without the loss of functional properties. Therefore, a possible alternative is to explore the genetic variability of wheat genome donors and wild cereal species. Several research groups (Jouanin et al., 2019; Juhász et al. 2018) demonstrated that alpha-gliadin sequences from the three genomes of hexaploid bread wheat contain different sets of T-cell stimulatory epitopes with unequal distribution. Similarly, differences in sequence composition and epitope content can be seen when gamma gliadins are compared (Shewry, 2018). Our previous study (Gell et al., 2015) showed that some einkorn varieties have lower R5 and G12 reactivity compared to bread wheat and other wheat genome donors. According to these findings, the aim of our project was now to analyse the proteins antigenic for celiac disease patients in a genetically heterogeneous large einkorn genotype collection.

In this study, 208 wild and domesticated einkorn genotypes were pre-screened with ELISA using celiac serum samples, and the storage protein composition of the genotypes with significantly lower immune reaction was further characterized. As a result, four candidate genotypes, MVGB770, MVGB1177, MVGB787 and MVGB748 with the lowest immune reactivity were selected for detailed proteomic analyses.

## 2. Materials and methods

### 2.1. Plant material and homogeneity test of the einkorn accessions

145 accessions of wild- and cultivated einkorn genotypes (*T. monococcum* L. ssp. *aegiloides* and *Triticum monococcum* L. ssp. *monococcum*) were obtained from the Department of Plant Genetic Resources and Organic Breeding (Agricultural Institute, Centre for Agricultural Research, Martonvásár, Hungary). From each accession five seeds were halved and halves containing the endosperm were used for homogeneity testing using gliadins extracts and one-dimensional gel electrophoresis. The other halves of the seeds containing the embryo obtained from 63 non-homogeneous accessions were grown in a greenhouse to full maturity. Altogether, 208 genotypes were screened with indirect serological ELISA assay. The detailed list of the genotypes derived from the Cereal Gene Bank is presented in the Supplementary Table 1. Bánkúti 1201, a hexaploid bread wheat cultivar was used as a control.

### 2.2. Patients and sera

Serum samples obtained from celiac patients with known HLA-DQ haplotypes and presence of celiac disease antibodies on gluten intake (n = 21, twelve females, nine males), median age 8.4 years, range 1.8–40.5 years) collected at Heim Pál National Paediatric Institute,

Budapest, Hungary were used in the study. Celiac disease was diagnosed by small intestinal biopsy showing Marsh III lesion combined with elevated IgA anti-transglutaminase (TG2) antibody serum levels. (Husby et al. JPGN 2020). Included patients had high anti-TG2 IgA levels (>100 U/ml) at the time of diagnosis. Serum samples from celiac patients adhering to a strict gluten-free diet (GFD) and with normalized antibodies (anti-transglutaminase IgA < 10 U/l) and mucosal healing (n = 3), and four healthy control subjects also, were used for immunoblotting studies. Based on the requirements of celiac disease related protein identifications all of the sera were used individually in this study both for ELISA and immunoblots. Altogether 17 serum samples (12 untreated, two treated celiac disease, and three healthy controls) were applied for the initial ELISA assays, 7 serum samples were used for 1D immunoblot (4 untreated, and 3 healthy controls). In the case of 2D immunoblot analyses, 12 serum samples were used (8 untreated, 1 treated celiac disease and 3 healthy controls) for the total protein immune detection of the MVGB770 genotype, while for the same analyses of the prolamin extracts 7 serum samples were applied (4 untreated, and 3 healthy controls). In case of MVGB1177, 748 and 787 2D immunoblots 7–7 serum samples were applied (4–4 untreated and 3 healthy controls).

### 2.3. Protein content and extractions

The crude protein content of the six selected einkorn samples (MVGB40, MVGB748, MVGB770, MVGB786, MVGB787 and MVGB1177, MVGB491) was determined by the Dumas method in triplicates, using a nitrogen conversion factor of 5.7 adaptation (AOAC, 1995) on an automated protein analyzer (LECO FP-528, USA).

Protein extraction for indirect serological ELISA screen was performed following the protocol of Dupont and co-workers (2011). Briefly, SDS-Tris extraction buffer (pH 6.8) containing 2% SDS, 10% glycerol, 50 mM DTT and 40 mM Tris-HCl, pH 6.8 was used to extract the total protein at room temperature for one hour with regular gentle vortexing. The extract was centrifuged at 16 000 g for 15 min (Sigma, 1–16 K). The supernatant was precipitated with four-volume of ice-cold acetone.

To obtain the salt/water-soluble proteins and gliadins/polymeric glutenins separately for the 2D gel electrophoresis (GE) and serology analyses, the samples were extracted using Osborne fractionation (Osborne, 1907) with little modification. First 0.5 M NaCl was used to extract the salt/water-soluble fraction at room temperature for one hour using gentle shaking. The extract was centrifuged at 16 000g for 15 min. The protein concentration was measured from the supernatant by (NanoDrop Spectrophotometer ND-1000 V3.8.1., Thermo Fischer Scientific). The pellet was further extracted using 70% ethanol followed by reduction (using 100 µl β-mercaptoethanol) and alkylation (with 200 µl 40% acrylamide). Finally, the obtained prolamin fraction was precipitated using 1.5 vol ice-cold acetone at –20 °C for 16 h. The precipitated proteins were washed three times with 1 ml ice-cold acetone, and dried in an Eppendorf Concentrator 5301 (Eppendorf, Hamburg, Germany), followed by a resolubilization step in sample buffer (4% SDS, 20% glycerol, 50 mM DTT, 120 mM Tris-Cl, 0.02% bromophenol blue, pH6.8) or IEF buffer (8 M urea, 2% CHAPS, 100 mM DTT (Dithiothreitol, Sigma-Aldrich), 0.2% CA (Carrier ampholyte, 40% BioLyte® 3/10) and 0.1% Bromophenol Blue) depending on the final application.

### 2.4. ELISA assay with patient samples

ELISA analyses were carried out using high-affinity binding plates in four replicates (Corning™, Costar™ 96-well assay plate). The ELISA screening study started with the optimization of different steps, like the sera- and antibody dilutions and blocking conditions. Cereal antigen fractions were further diluted to 10 µg/ml in sodium bicarbonate coating buffer (100 mM Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub>, pH9.6) to obtain solubility and added to the plate for overnight at 4 °C. Bread wheat was used as control. After extensive washing with PBS-TWEEN buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>), plates were blocked for 1 h with 5%

casein hydrolysate and 0.05% TWEEN20 and then incubated with human patient serum samples diluted 100x in PBS buffer. After further washings, the binding was detected with anti-Human IgA ( $\alpha$ -chain specific) peroxidase - conjugated antibody (diluted 1:5000) produced in goat (Sigma-Aldrich-A0295) in the presence of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, Missouri, United States). Absorbance was read at 450 nm after stopping the reaction with 2 N H<sub>2</sub>SO<sub>4</sub>. For serological ELISA human sera of fourteen HLA DQ 2.5 and seven HLA DQ8 patients of different age and gender were used as listed in Supplementary Table 2. This custom-developed ELISA method was adopted from the study of Sharma et al. (2016).

## 2.5. RIDASCREEN R5 and ROMER G12 sandwich ELISA tests

R5 Ridascreen Gliadin (R7001, R5 monoclonal antibody, sandwich format, LoD: 0.5 mg/kg gliadin or 1 mg/kg gluten, LoQ: 2.5 mg/kg gliadin or 5 mg/kg gluten, R-Biopharm, Germany) sandwich enzyme immunoassay and the AgraQuant Gluten G12 (COKAL0200, G12 monoclonal antibody, sandwich format, LoD: 2 mg/kg gluten, LoQ: 4 mg/kg gluten, Romer Labs, Austria) sandwich enzyme assay were used to determine the R5 and G12 monoclonal antibody responsive of the prolamins extracts. Prolamin extraction and dilution of einkorn samples was performed in four replicates according to the manufacturer's instructions. Einkorn extracts were diluted to 1:500 (in case of R5), and to 1:400 (in case of G12) final concentrations to ensure sufficient sensitivity even at lower protein levels. ELISA assays were performed as outlined in the manuals provided by the manufacturers; the cubic spline algorithm was used for the standard curve construction. Results were corrected by the dilution factor used for the flour samples.

## 2.6. 2D gel electrophoresis and immunoblotting

2D GE was performed according to Görg et al. (2007), and started with an extensive optimization of the IEF buffer composition, the isoelectric focusing profile settings and acrylamide gel density. The precipitated protein fractions were solubilized in IEF buffer and separated by isoelectric focusing in three replicates per sample for nanoLC-MS/MS analyses and as much as needed for western blot analyses, depending on the number of sera used for. The IEF was carried out in 7 cm Immobiline DryStrips pH 3–10 (GE Healthcare), under overnight rehydration using 200  $\mu$ g proteins in 150  $\mu$ l IEF buffer. The second dimension was carried out on 15% polyacrylamide gels in Hoefer™ Mighty Small™ II Deluxe Mini Vertical Electrophoresis Unit (Hoefer Inc., USA). 2D GE analysis was made in three technical replicates, on 15% polyacrylamide gels. The blot conditions, like blotting time, voltage, the sera and antibody dilutions and the development were also optimized. After the 2D GE, proteins were transferred to an ImmobilonP PVDF membrane (Millipore, Billerica, USA) and blocked for 1 h with 5% casein hydrolysate and 0.05% TWEEN20 followed by overnight incubation at 4 °C with 1:20 diluted blood sera. The immune-reactive proteins were detected with anti-Human IgA peroxidase-conjugated antibody produced in goat (Sigma-Aldrich-A0295) in the presence of 4-Chloro-1-Naphthol chromogenic peroxidase substrate. All of the immune responsive protein spots from the three technical replicates of the parallel run 2D GE gels were excised and the bulked spots were sent for protein identification using nano-LC-MS/MS.

## 2.7. Peptide-specific antibody (DG4) purification

500  $\mu$ g of biotinylated synthetic dodecapeptide with sequence SGGPLQPQPPF, (ThermoFisher) was immobilized to 1 ml settled Pierce™ High Capacity Neutravidin Agarose (Thermo Scientific, Rockford, Illinois, USA) according to the manufacturer's instructions. Celiac patient serum was diluted two times in PBS + 0.1% Tween and incubated with the peptide bound agarose for 1 h at room temperature. The antibodies were eluted with 5 column volume of 100 mM glycine pH

2.5 followed by buffer change to PBS with 50 K Amicon® Ultra Centrifugal Filters (Merck, Darmstadt Germany). The purified DG4 fraction contained approximately 90% IgG and 10% IgA antibodies as measured by ELISA with the target peptide.

ELISA assays were carried out as detailed in previous section. DG4 antibodies were diluted 1000x and 6000x in PBS buffer complemented with 0.5% casein hydrolysate. After washing the binding was detected with anti-Human IgA ( $\alpha$ -chain specific) (Sigma-Aldrich-A0295) (1:5000) or IgG ( $\gamma$ -chain specific) (Sigma-Aldrich-A6029) peroxidase conjugated antibodies, respectively, produced in goat (1:6000) in the presence of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, Missouri, United States).

1D and 2D immunoblot analyses of the einkorn prolamins and water-soluble extracts with DG4 antibodies were carried out as detailed previously. DG4 primary antibodies were diluted 1000x, detected with anti-Human IgA ( $\alpha$ -chain specific; Sigma-Aldrich-A0295) and 6000x, detected with anti-human IgG ( $\gamma$ -chain specific) (Sigma-Aldrich-A6029) peroxidase-conjugated antibodies. IgA secondary antibodies were applied in 1:5000 dilutions in case of salt-water fraction and in 1:6000 dilutions in case of prolamins fraction. IgG secondary antibodies were applied in 1:6000 in the case of both salt-water and prolamins fractions. 3,3',5,5'-Tetramethylbenzidine (TMB) ready to use solution Liquid Substrate System for Membranes were used for detection. The reaction was stopped after 5 min using PBS buffer.

## 2.8. Protein identification by nano-LC-MS/MS

Gel spots were in-gel digested for 4 hrs at 37 °C with sequencing grade modified porcine trypsin (Promega) after reduction with DTT and alkylation with iodoacetamide (Sigma-Aldrich). The peptide extracts were analyzed using on-line nano-LC-MS/MS technique on a Waters nano-Acquity UPLC (Waters Corporation, Waters USA) coupled with Thermo LTQ-Orbitrap-Elite mass spectrometer (Thermo Fischer Scientific, USA). Ion-trap CID spectra were acquired from the ten most abundant peaks after each survey scan. Proteome Discoverer (Thermo) was used for generating MSMS peak lists and in-house Protein Prospector, Ver 6.1.10 was used for database search against the whole SwissProt.2019.6.12 (560292/560292 entries searched) random concat database complemented by the Tricomic sequences of the UniProt database: UniProtKB.2019.6.12. random.concat (369503/158817814 entries searched) and appended with a *Triticum monococcum* specific dataset built from public einkorn seed transcriptome datasets (SRX283514/SRR924098 (DV92) and SRX257915/SRR922411 (G3116), (Fox et al., 2014) (123892/123892). The following search parameters were used for tryptic peptides: carbamidomethyl-Cys as constant, oxidation of Met, pyro-Glu from peptide N terminal Gln and protein N-terminal acetylation as variable modifications. Only fully tryptic peptides were considered with a maximum of 1 missed cleavage site. Mass tolerance was set to 5 ppm for the survey and 0.6 Da for the MS/MS measurements respectively. Minimum protein and peptide score were 51 and 20 respectively as acceptance criteria. In case of homologous proteins, the one with the highest protein score (above 50) and larger sequence coverage was used. Proteins with a number of unique peptides above two were listed in the analyses. At the final selection process redundant proteins were excluded from the identified protein list. The immune reactive peptide hits were visualized using the Motif Search algorithm of CLC Genomics Workbench v7.6.4 (Qiagen, Aarhus, Denmark) and mapped to the final selected protein sequences with 100% sequence identity.

## 2.9. Size Exclusion-High performance liquid chromatography (SE-HPLC)

SE-HPLC was used to determine the glutenin, gliadin and albumin + globulin contents, using a modification of the Batey et al. (1991) method, as described in Rakszegi et al. (2017).

Ten milligrams of flour in three replicates were suspended in 1 ml

0.5% (w/v) SDS in phosphate buffer (pH 6.9) and sonicated for 15 s. After centrifugation, the supernatant was filtered on a 0.45  $\mu\text{m}$  PVDF filter. Analyses were performed on a Phenomenex BIOSEP-SEC 4000 column (300x7.8 mm, 5  $\mu\text{m}$ , 500  $\text{\AA}$ ) in acetonitrile buffer [0.05% (v/v) trifluoroacetic acid and 0.05% (v/v) acetonitrile] with a running time of 10 min (2 ml/min flow rate). Proteins were detected by absorption at 214 nm.

### 2.10. Reversed-phase high performance liquid chromatography (RP-HPLC)

The einkorn flour (60 mg) was extracted using 70% ethanol and vortex for 30 min in a horizontal vortex, (MO BIO Laboratories, Inc. Vortex-Genie® 2). Samples were centrifuged for 15 min at 13,000 rpm using an Eppendorf Centrifuge 5424. The supernatant was filtered using a 0.45  $\mu\text{m}$  filter into an HPLC glass vial. The protein extracts were separated using an Agilent 1200 LC system (Agilent Technologies) using the method of Larroque et al., 2000. 10  $\mu\text{l}$  of extract were injected into a C18 reversed-phase Zorbax 300SB-C18 column (4.6  $\times$  150 mm, 5  $\mu\text{m}$ , 300  $\text{\AA}$ , Agilent Technologies) maintained at 60°C. The eluents used were ultrapure water (solvent A) and acetonitrile (solvent B), each containing 0.1% TFA (Trifluoroacetic acid, HPLC grade, Sigma Aldrich). The flow rate was adjusted to 1 ml /minute. Proteins were separated using a linear gradient from 21% to 47% of solvent B in 55 mins and detected by UV absorbance at 210 nm. Each sample was sequentially injected three times for technical replication. RP-HPLC peak areas (expressed in arbitrary units, AU) under the chromatograms were used to calculate gliadin amounts;  $\omega$ -gliadins were considered between 15 and 30,  $\alpha$ -gliadins between 30 and 40 and  $\gamma$ -gliadins 40–55 min (Marchylo et al., 1988).

### 2.11. Sequence analyses and epitope mapping

Peptides identified from the LC-MS/MS analysis of the prolamin extracts were used for peptide mapping to investigate gliadin-type specific variations. Peptide sequences containing missed cleavages,

ragged ends and modifications have been discarded from the analysis. Celiac disease-specific linear T-cell core epitopes and wheat peptides with reported strength of immune response (Tye-Din et al., 2010; Juhász et al., 2018; Sollid et al., 2020) were mapped to the identified protein sequences. A threshold of 100% sequence identity was used to identify wheat epitope homologues in the einkorn protein hits. Results were visualized using the Morpheus R package (<https://software.broadinstitute.org/morpheus/>).

### 2.12. Statistical analyses

ANOVA analyses completed with the Tukey test, and multiple comparisons of mean values were carried out as implemented in the OriginPro 2021 Statistical Software (OriginLab Corporation, 2021); significance levels were set to  $p < 0.05$ .

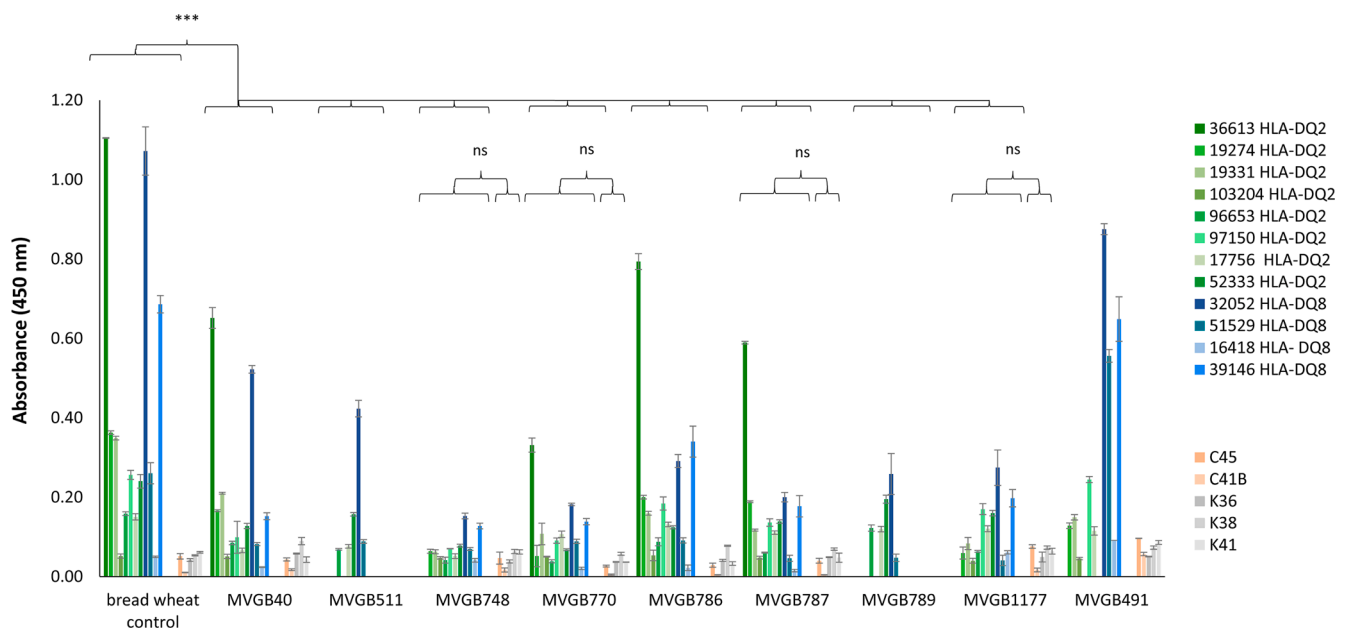
## 3. Results

### 3.1. ELISA screening with celiac sera

In our study a comprehensive analysis was conducted using the Martonvásár Cereal Gene Bank einkorn collection consisting of 208 einkorn accessions. Altogether 17 serum samples (12 with untreated and 2 with treated celiac disease, and 3 healthy controls) were applied for the initial ELISA assays. Cohorts differed in gender, age and HLA-DQ haplotypes. Eight of the 208 einkorn genotypes (MVGB40, MVGB511, MVGB748, MVGB770, MVGB786, MVGB787, MVGB789 and MVGB1177) showed significantly lower antibody response compared to the hexaploid bread wheat control (cv. Bánkúti 1201;  $p < 0.0001$ ) (Fig. 1).

The genotypes MVGB511, and MVGB789 were analyzed with five sera (96653-DQ2.5; 17756-DQ2.5; 52333-DQ2.5; 32052-DQ8 and 51529-DQ8). The overall layout of the experiments is shown in Supplementary Fig. 1.

Two einkorn genotypes, MVGB770 (14.21%) and MVGB748



**Fig. 1.** Absorbance values (450 nm) of total seed storage protein extracts of selected *Triticum monococcum* L. ssp. *monococcum* genotypes MVGB770, MVGB1177, MVGB748, MVGB787, MVGB786, MVGB40 and MVGB491 in ELISA with serum samples from celiac disease patients with active disease and either HLA-DQ2 or HLA-DQ8 background, celiac patients adhering to a strict gluten-free diet (GFD) (C45, C41B), and healthy controls (K36, K38, K41) measuring IgA antibodies. Hexaploid bread wheat cv. Bánkúti 1201 was used as a control. \*\*\* $p < 0.001$  in the indicated einkorns versus hexaploid wheat for the serum samples with active celiac disease by ANOVA tests, \* $p < 0.05$  in hexaploid wheat for active celiac disease samples versus treated celiac or non-celiac control serum samples. Similarly,  $p < 0.05$  was observed for MVGB491 in the reactivity of 9/12 celiac serum samples with active disease versus treated and non-celiac control samples. There was no significant difference between einkorns and hexaploid wheat in the reaction of treated (seronegative) celiac patients or non-celiac control subjects.



**Table 1**  
Summary of results obtained from ELISA screen, R5 and G12 ELISA tests and HPLC measurements.

MVGB einkorn genotypes	40	511	748	770	786	787	789	1177	Hexaploid wheat control
CD IgA ELISA reactivity (%DQ2 patients (n = 8))	38.67–98.06%	43.31–65.34%	26.00–90.97%	14.21–96.77%	45.70–71.88%	33.52–92.26%	77.13–81.26%	16.34–80.04%	100%
DQ8 patients (n = 4)	22.16–48.69%	34.05–39.46%	14.24–82.83%	16.95–41.41%	27.15–49.56%	17.68–30.30%	18.13–24.10%	15.66–25.59%	100%
Gluten reactive with R5 Ridascreen ELISA (ppm)	22.45		32.29	36.05	36.48	35.91	33.33	33.33	393.095
Gluten reactive with G12 AgraQuant ELISA (ppm)	190.60		190.31	186.53	198.57	190.04	197.36	197.36	225.408
<b>Total gliadins by HPLC (g/100 g flour)</b>	9.53			7.93	8.95	9.08			9.83
	96.94%			80.67%	91.04%	92.37%			
Absolte alpha-gliadin content (g/100 g flour)	5.46			4.75	4.63	4.97			5.52
Absolute gamma gliadin content (g/100 g flour)	2.77			2.82	3.46	3.00			3.89
Absolute omega gliadin content (g/100 g flour)	1.3			0.35	0.86	1.11			0.42
<b>Total glutenins by HPLC (g/100 g flour)</b>	7.02			10.09	8.11	8.18			9.12
	76.97%			110.6%	88.92%	89.69%			100%
<b>Albumin + Globulin fraction by HPLC (g/100 g flour)</b>	1.61			1.50	2.01	1.80			1.46
	110.66%			103.07%	138.09%	123.62%			100%

§Values in italics indicate % of hexaploid wheat control, parameters in empty cells were not measured. p values were < 0.0001 for RP-HPLC, R5 and G12 sandwich ELISA, p < 0.05 in case of CD IgA ELISA and < 0.05 in case of the SE-HPLC measurements.

(10.24%) resulted in the lowest signal with the performed serological ELISA assays when compared to the bread wheat control using twelve different celiac and the two control sera. Using individual HLA DQ2 patient sera 14.21–98.06% of bread wheat response was measured, while the use of individual HLA DQ8 sera resulted in 14.24–82.83% immune response of that of the bread wheat control. The MVGB491 was the most immunogenic einkorn genotype of our collections with 95.44–213.74% values of bread wheat control. After excluding MVGB491, the average response with all of the applied DQ2 sera was the lowest against MVGB748 (35.27%) and in the case of DQ8 sera the lowest immune response had MVGB789 with 21.11%, compared to bread wheat control. Considering all sera used for ELISA measurements the lowest response was measured from MVGB770 with 34.73% in average. The immune response against the total protein extracts of MVGB770, MVGB748 and MVGB787 was below 40% compared to the bread wheat control. The data of the measured immune responses are summarized in Table 1.

Based on the serological ELISA, 1D immunoblot analysis was conducted using the eight lowest immune signal giving genotypes (shown in Supplementary Fig. 2) and based on it six genotypes were selected for R5 and G12 sandwich ELISA analyses.

### 3.2. R5 and G12 ELISA

The ELISA assays of six selected einkorn genotypes showed low immune reactivity with the celiac serum samples compared to the bread wheat control. The analysis was supported by R5 Ridascreen ELISA and showed significantly lower levels of R5 mAb specific responses in all of them. Using a 500x dilution factor in the evaluation of the R5 ELISA results each of these six cultivars resulted in < 100 ppm gluten content, (22.45 ppm – 36.48 ppm gluten (Fig. 2)).

In the case of hexaploid wheat, the R5 ELISA test required a higher dilution of the protein extract, to provide accurate and interpretable results within the detection limits, and as a positive control, resulted in 393095 ppm gluten content.

In the G12 ELISA assay, we used a final dilution of 1:400 (10x dilution factor at the kit calculation) to measure the monoclonal antibody response against the einkorn genotypes. The results varied between 187 and 199 ppm gluten. Higher dilution factor (10 000x) was needed to measure the G12 mAb response in the bread wheat control, and 112704 ppm gliadin and 225408 ppm gluten were measured (Fig. 2). Based on the ANOVA the results of the R5 and G12 ELISA are significant (p < 0.0001).

Summarizing the results of the celiac serum and commercial gluten ELISA analyses, four genotypes were selected for further HPLC and immunoblot analyses.

### 3.3. SE- and RP-HPLC

Protein composition of the einkorn and bread wheat control samples has been characterized with SE-HPLC, followed by the RP-HPLC analyses of the gliadin fraction. SE- and RP-HPLC showed remarkable variability in the absolute gliadin contents in einkorns (calculated in g gliadin/100 g flour (Supplementary Figs. 3 to 5)). According to the SE-HPLC analyses (Table 1 and Supplementary Fig. 4.) the MVGB770 genotype contains the lowest amount of total gliadin value and the MVGB40 genotype has the highest absolute total gliadin content, with values 80–97% of the hexaploid wheat control. The glutenin fraction in einkorns varied between 7.02 and 10.09 g in 100 g flour (p < 0.05 by ANOVA compared to bread wheat). The absolute amounts of the albumin/globulin proteins were higher in all of the einkorn genotypes than in the bread wheat control (p < 0.05).

Based on the gliadin composition measured by RP-HPLC, in each genotype the alpha gliadins contribute the greatest level to the total gliadin content with values between 51.75% and 59.92%. Gamma gliadin % resulted between 29.06 and 38.61% while the omega gliadin

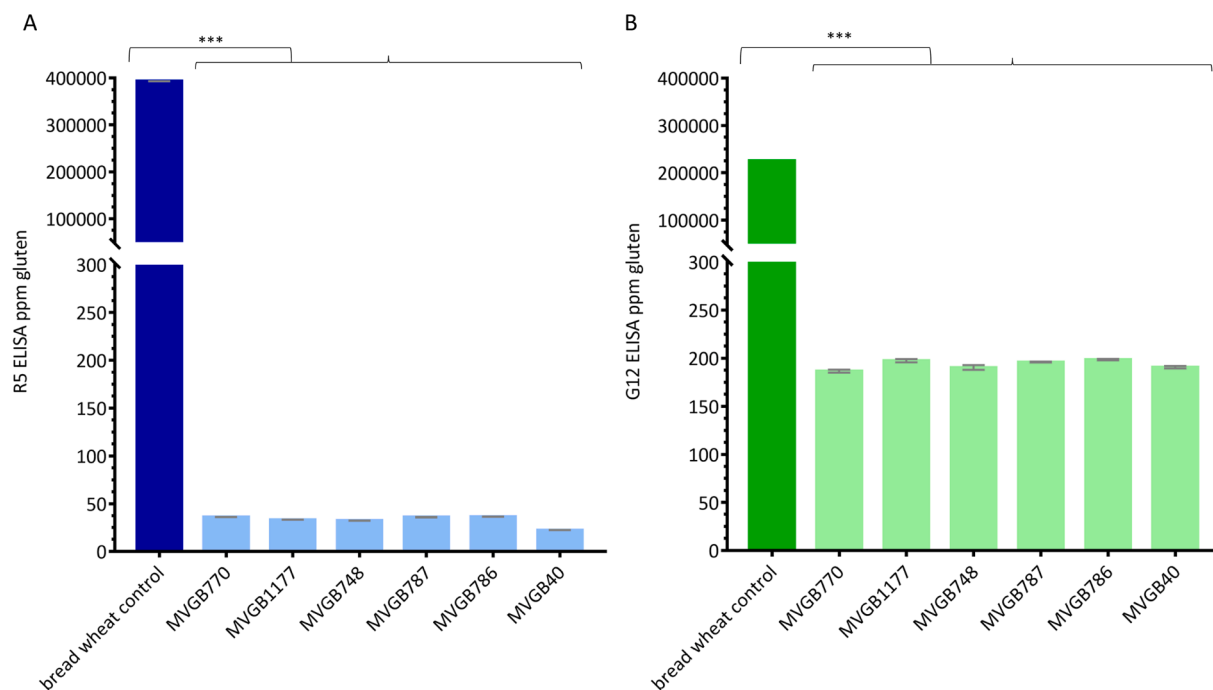


Fig. 2. Gluten content (ppm) of selected *Triticum monococcum* L. ssp. *monococcum* genotypes MVGB770, MVGB1177, MVGB748, MVGB787, MVGB786, MVGB40 and bread wheat control based on R5 and G12 ELISA assay results, \*\*\* $p < 0.0001$  compared to bread wheat, no significant difference between einkorns.

content was the lowest (4.47%–13.63%). Together, these results indicate that the low reactivity with the R5 and G12 antibodies is related to the different gliadin/gluten sequences in the einkorns and not to the low absolute amount of prolamins.

### 3.4. Protein identification by nano-LC-MS/MS

The genotype MVGB770 served as a representative for the immune reactive protein set based on the highest number of immune responsive proteins detected from the total protein extracts and was used to identify these proteins.

There were 110 protein spots identified from the total protein extract of MVGB770. Altogether 16 protein spots were immune-reactive with celiac disease antibodies (Fig. 3) and were further analyzed by on-line nanoLC-MS/MS. No immune reactive proteins were detected when sera of healthy controls or patients on a strict gluten-free diet were applied (Supplementary Table 3). Several groups of non-gluten proteins, including serpins, alpha-amylase/trypsin inhibitors (ATI), 1S- and 12S storage globulins were also detected besides the major gluten proteins. In addition, celiac sera (with anti-human IgA) showed affinity to several enzymes, such as beta amylase malate dehydrogenase, alanine amylo-transferase, formate dehydrogenase, glucose-1-phosphate dehydrogenase, peroxidase, aldose reductase, UDP arabinopyranose mutase and glutamate dehydrogenase. Characteristics of predominant immune-reactive protein hits including their protein scores, the number of unique peptides and the sequence coverage are presented in Supplementary Table 4.

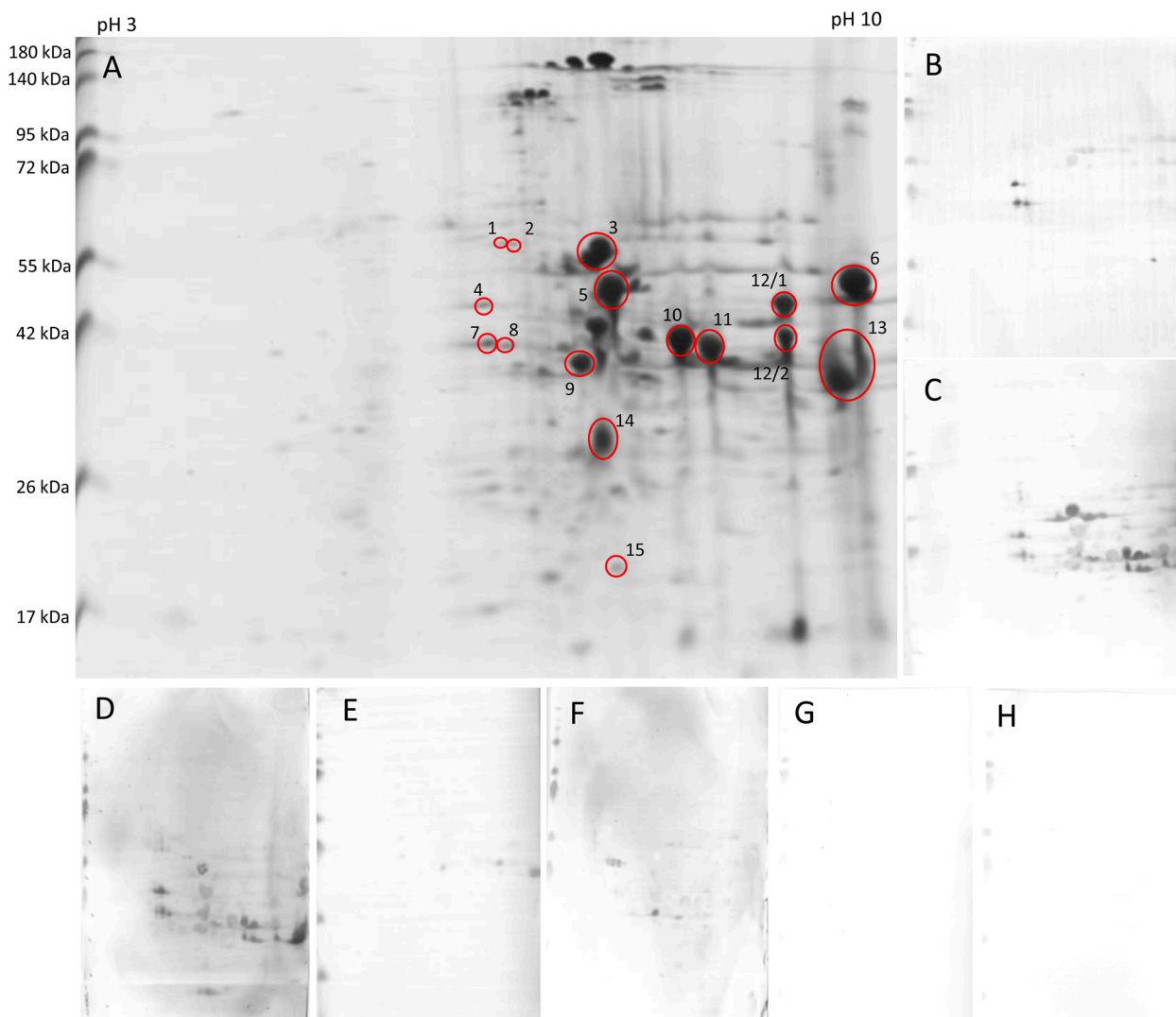
The prolamins extracts of the most promising four selected einkorn genotypes (MVGB770, MVGB1177, MVGB787 and MVGB748) were also analyzed in similar ways, (Fig. 4, Supplementary Fig. 6, Supplementary Tables 5 and 6).

Sequence analyses were carried out to compile an accurate list of the celiac related immune reactive prolamins present in the selected einkorn genotypes. In all of the genotypes but MVGB748 gamma gliadins, LMW- and HMW glutenins, and alpha gliadins have been identified. The detailed list and characterization of identified protein hits for each spot separately are shown in Supplementary Table 6.

Fully tryptic peptides identified from the mass spectrometry analysis of prolamins extracts of the four genotypes (MVGB748, MVGB770, MVGB787 and MVGB1177) were mapped to the identified protein sequences along with known B- and T cell epitopes (Sollid et al., 2020). Next to alpha and gamma gliadins, LMW and HMW glutenin sequences, an avenin-like protein and an alpha-amylase trypsin inhibitor were also detected in the immune reactive protein set. T-cell epitope mapping results show a significant variation in the number of alpha gliadin sequences: seven alpha gliadins have been identified in the MVGB787, six alpha gliadins in the MVGB770 and only one alpha gliadin has been identified in the MVGB1177 and MVGB748 genotypes, respectively. While proteins identified from the MVGB787 and MVGB770 genotypes contain the DQ2.5- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 3 epitopes, these were absent in the proteins identified from the two low (MVGB748, MVGB1177) immune responsive genotypes. The number of detected gamma gliadins, LMW, and HMW glutenins showed fewer variations, and only two gamma gliadin proteins have been identified in MVGB748 (Fig. 5). B-cell epitope mapping also shows high differences in the epitope content of the protein hits, especially one gamma-gliadin (P04729) and an avenin-like protein (V5M0Y3) contains only one B-cell epitope IPEQ. From the protein hits an alpha-gliadin (A0A0E3Z7R8) contains 95 different B-cell epitopes, as the most immune reactive protein from the detected collection (Supplementary Fig. 7).

To further characterize the celiac epitopes in einkorns, antibody (DG4) affinity-purified from the serum sample of patient 83010 (DQ8/X) by the synthetic QPQQPFP gamma gliadin peptide sequence were utilized, since its deamidated form (QPEQFPF) is one of the major epitopes in clinical diagnostic investigations for celiac disease. The DG4 antibodies were reactive with all selected einkorns, however this response was lower in indirect ELISA compared to bread wheat both for anti-IgA (51.4–83.8%) and anti-IgG (28.3–58.6%) detection and DG4 recognized distinct protein bands in immunoblots (Supplementary Fig. 8, Supplementary Table 7). The densitometric values of the DG4 reactive protein bands are presented in Supplementary Fig. 9.

The most immunoreactive B-cell epitope gamma-gliadin sequence QPQQPFP (Schwertz et al., 2004) was detected in the protein hits of MVGB770, MVGB1177 and MVGB787, but fully absent in MVGB748



**Fig. 3.** Identification of celiac IgA reactive proteins of the total protein extract of einkorn MVGB770. (A) 2D SDS polyacrylamide gel electrophoresis of total protein extract of MVGB770. Protein spots were separated on 7 cm pH 3–10 IPG strips followed by the separation on 15% acrylamide gels. The spots labelled with red circles represent celiac IgA reactive proteins in the corresponding immunoblots which were subjected to on-line nano LC-MS/MS analyses. Molecular weight range is labelled on the left side in kDa. (B–F) Immunoblots with human sera and anti-human IgA secondary antibodies (B, C, D active celiac disease with HLA DQ2 genetic background; E, F active celiac disease with HLA DQ8). (G) Healthy control and (H) celiac disease patient on gluten free diet. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 5). MVGB748, however, also showed a detectable signal in ELISA and immunoblot studies with DG4. It contained shorter or modified versions of this sequence in the protein hits, like QPQPQQ, QQQPQQF, QPQQ(L/Q), QQP(S/Y). The 2D GE western blot of MVGB770 and MVGB748 prolamins extracts with DG4 antibody results showed the same immune responsive protein patterns as in immunoblots with celiac sera. Based on the nanoLC-MS/MS peptide list, these proteins belong to gamma and alpha gliadins and low molecular weight glutenins (spots 8, 9, 10, 12, 13 and 14 of MVGB770 in the Supplementary Table 6).

#### 4. Discussion

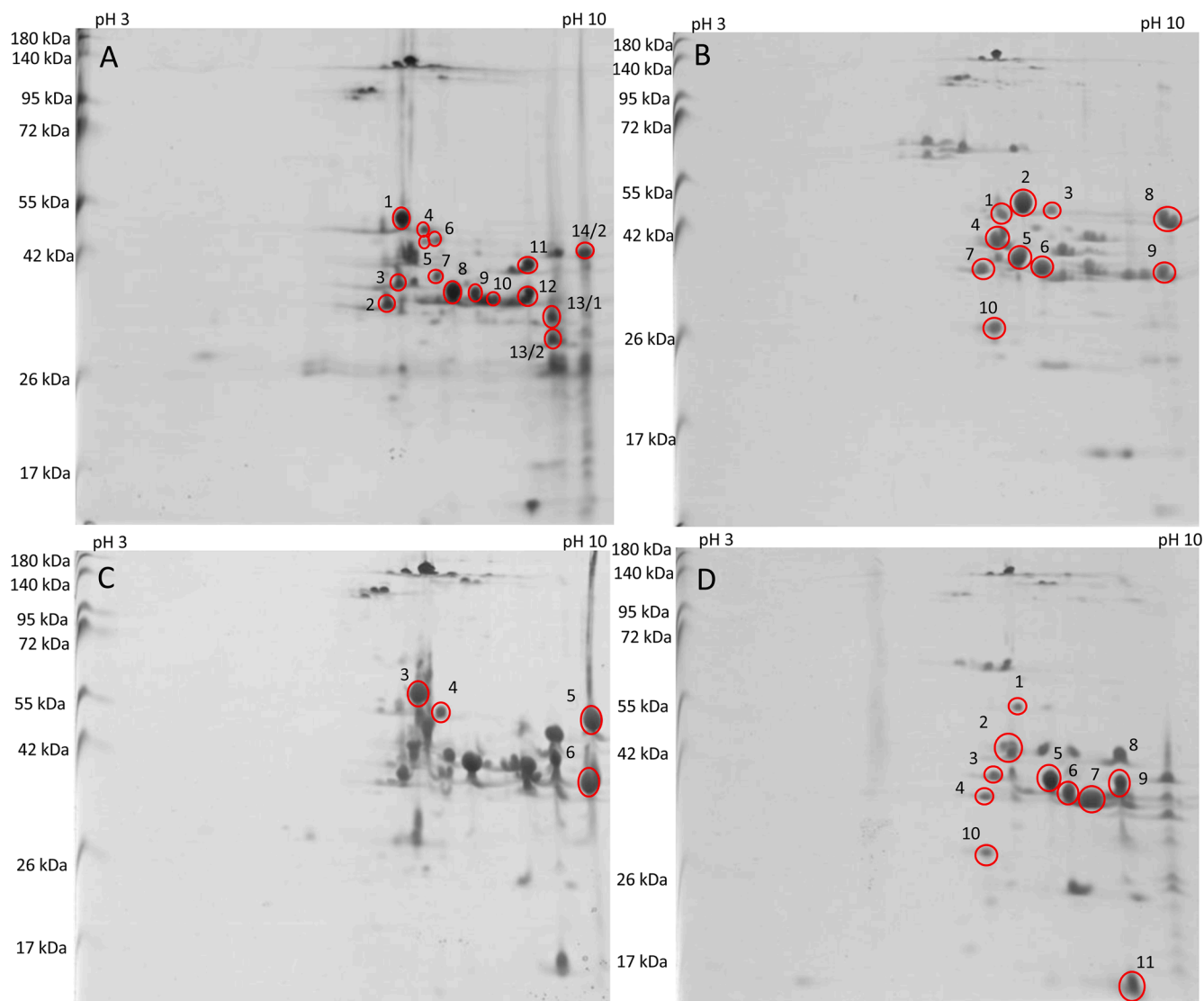
Our work aimed to carry out an immune analytical screening of an einkorn collection of 208 cultivated- and wild einkorn genotypes maintained at the Cereal Gene Bank of the Agricultural Institute in Martonvásár, Hungary. Previous reports on einkorn focused on their characterization as a potential new dietary food source for celiac patients, using variable clinical and proteomic studies (Vaccino et al., 2009; Gianfrani et al., 2012; Ozuna et al., 2015; Iacomino et al., 2016; Geisslitz et al., 2019; Picascia et al., 2020; Di Stasio et al., 2020). Most of

these studies reported results based on the analysis of a limited number of einkorn genotypes, while our workflow was built on an extensive screening of a wide einkorn population with the purpose to detect the least immune reactive genotypes without clinical trial.

Identification of new prolamins alleles of wheat relatives and wild wheat species is of great importance in order to find germplasm for special end-use quality purposes as well as development of food with reduced toxicity (Juhász et al., 2012; Juhász et al., 2018; Gell et al., 2015).

Our large-scale pre-screening examination of a highly diverse initial einkorn population has been performed using celiac positive patient sera on total storage protein extracts. In each case only individual sera were used, which indicates a high diversity in the individual sensitivity of celiac patients. The high genetic variability of this einkorn collection was reflected in the significant diversity in immune reactivity. In some cases, the measured reactivity was equal or higher than the bread wheat control, which can be explained by the sequence diversity in the genotypes and the individual patient's susceptibility.

Despite the lower immune response, based on the HPLC analysis results these genotypes contained similar amounts of alpha gliadins as in



**Fig. 4.** 2D polyacrylamide gel electrophoresis of the prolamin fraction of four *Triticum monococcum* L. ssp. *monococcum* genotypes (A) MVGB770, (B) MVGB1177, (C) MVGB748, (D) MVGB787. Protein spots were separated on 7 cm pH 3–10 IPG strips followed the separation on 15% acrylamide gels. Protein spots labelled with red circles were reactive in corresponding immunoblots with serum IgA from celiac patient with active disease, and were sent to nano LC-MS/MS analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bread wheat. This finding was also confirmed by recent studies by Geisslitz et al., 2019 and Di Stasio et al., 2020. It indicates that the lower immune response can be at least partially related to the differences in the characteristic gluten protein sequences. In the case of MVGB770, the difference between the results obtained by G12 ELISA and SE-, RP-HPLC can be explained by the special alpha gliadin sequence composition, containing alpha gliadins without or with low amounts of known T- and B-cell stimulating epitopes.

The RP- and SE-HPLC examination demonstrated low diversity in the quantitative composition of prolamin fractions of the final selected four genotypes; the sandwich ELISA assays indicated the quite similar celiac antigenicity response of the assorted einkorn genotypes. This could be explained by the close relationship between the selected genotypes primarily representing the subspecies *T. monococcum* ssp. *monococcum* var. *vulgare*.

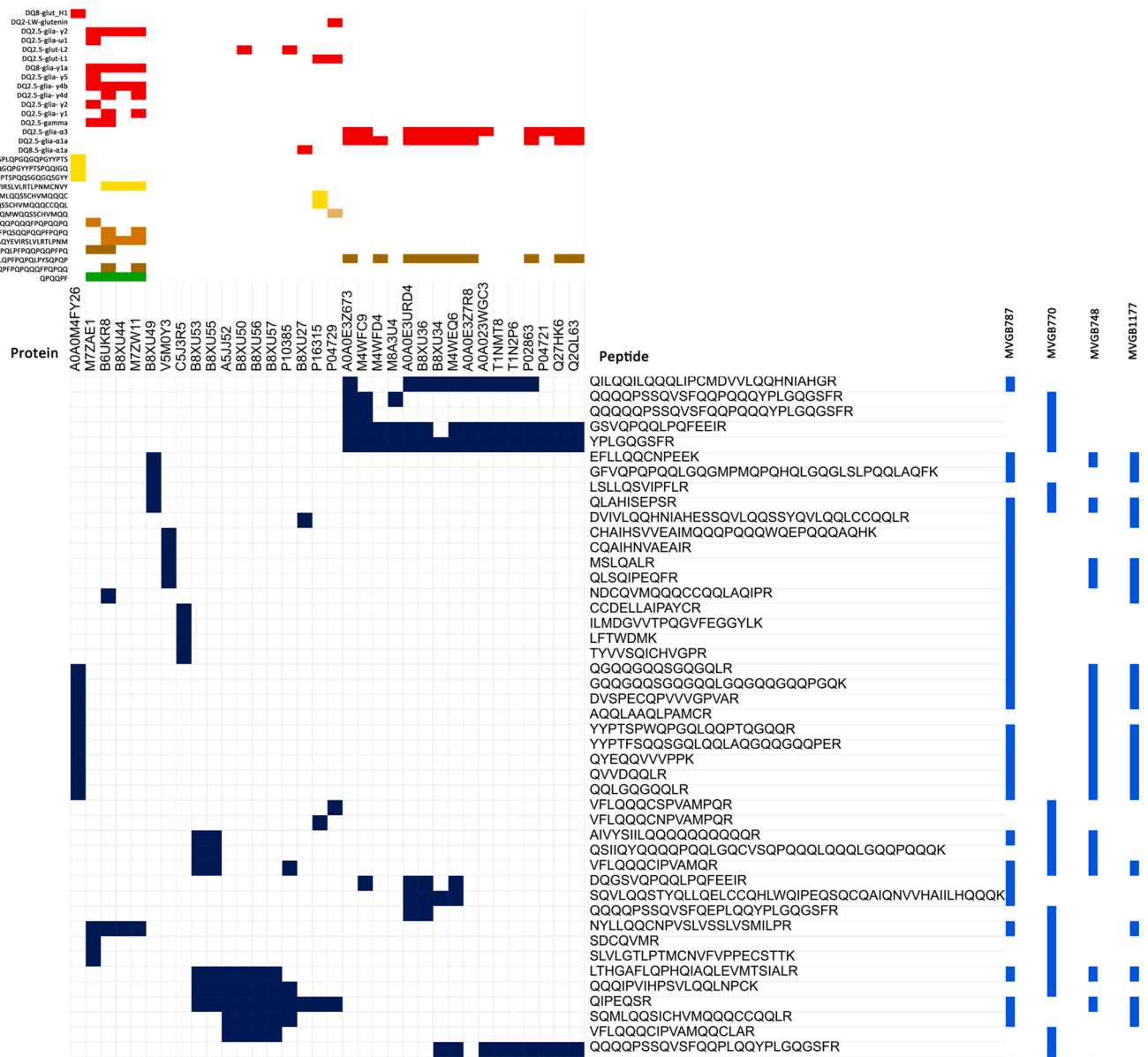
In contrast to bread wheat, the R5 and G12 mAb based ELISA results showed diverging quantitative results in the analysed einkorns. The R5 mAb was developed against the peptide QQFPF, characteristic in rye secalins, barley hordeins and wheat gliadins. R5 also recognizes homologous peptides such as LQFPF, QLPYP, QLPTF, QQSFP, QQTFP,

PQFPF and QQPYP, although with weaker reactivity (Valdés et al. 2003). The monoclonal antibody G12 was developed against the QPQLPY peptide, (Morón et al., 2008).

These commercial sandwich ELISA measurements indicated that einkorns contain four times more G12 reactive prolamins than R5 reactive sequences, which could be explained by the different target sequence of the antibodies. In the R5 ELISA assay, our pre-selected einkorn genotypes yielded only 22.45–36.47 ppm R5 reactive protein of the above mentioned epitopes, which sets 0.0057–0.0093% of the hexaploid bread wheat control. However, the HPLC measurements support the high gluten content of these einkorn samples.

Summarizing the results of the immunanalytical analyses none of the examined einkorn genotypes proved to be harmless for celiac disease patients, but their R5 reactive protein content (< 100 ppm) measured by the ELISA kits may represent a lower risk in combination with enzymatic treatments for patients suffering from other clinical conditions like non-celiac wheat sensitivity. Furthermore, modern food processing technologies can adjust certain immunoreactive components of wheat by applying malting and germination enzymes, fermentation and microbial enzymes (Kucek et al., 2015).





**Fig. 5.** Mapping results of prolamins peptides identified in the four genotypes. Heat map shows the peptides mapped to the identified proteins. T cell epitopes and epitopes with known levels of immune response present in the protein sequences are highlighted with red blocks. Peptides detected in the four genotypes are annotated in blue in the right hand-side panel. One of the most harmful B-cell and T-cell peptide (QPQQPF) is highlighted in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Similar to our findings other studies also confirmed that einkorn has reduced immunogenic level and a lower capability to trigger celiac disease (Vaccino et al., 2009; Gianfrani et al., 2012; Ozuna et al., 2015; Iacomino et al., 2016; Geisslitz et al., 2019; Picascia et al., 2020).

The final experimental step in our study was the 2D immunoblot and nano LC-MS/MS analysis of the four selected genotypes. The results of the immunoblot analysis using celiac patient samples and protein identification revealed that compared to the control wheat sample significantly lower immunogenicity is characteristic to each einkorn accession independent of the level of patient susceptibility to the analyzed protein. Consequently, ten times longer detection time was needed to develop visible signals on the blot membranes of einkorn protein extracts compared to the bread wheat. It indicates that IgA of celiac patients shows lower binding affinity to storage proteins in einkorn.

Our results confirmed that in addition to the well-recognized IgA response to prolamins fraction, celiac disease is also associated with

proteins with non-storage function, such as serpins, alpha-amylase trypsin inhibitors and globulins. These findings are in agreement with previous reports from wheat and other cereals (Huebener et al., 2015; Gell et al., 2017; Altenbach et al., 2020).

Moreover, several enzymes like beta-amylase, malate dehydrogenase, aminopeptidase, aldehyde-dehydrogenase, glucose-1-phosphate adenylyl transferases, alanine aminotransferase, glutamate dehydrogenase, peroxidase, aldose reductase and catalase have also been detected in almost every reactive protein spot.

Reaction with non-prolamins components could be explained in two different ways in celiac patients: either by sequence cross reactivity or by a secondary immune response during the disease process where long-standing intestinal damage may result in increased gut permeability and immune response to additional antigens. In order to dissect these mechanisms, we utilized affinity-purified patient antibodies (DG4) to the main antigenic QPQQPF gliadin sequence. Our results indicate that target or cross-reactive epitopes to this sequence are present, albeit in

much reduced amounts.

In case of celiac disease the specific B- and T cell epitopes have high sequence similarity and overlap with each other. The measured B cell immune response of the peripheral blood sera together with the nano LC-MS/MS identified proteins served a good base for the *in silico* B and T cell epitope mapping.

The epitope and peptide mapping analysis results indicated that the alpha-gliadin sequences in the monococum genotypes either completely lack the known T cell epitopes or only include the DQ2.5-glia- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 3 epitopes (Fig. 5). A detailed epitope mapping study of the bread wheat reference genome using T cell core epitopes and peptides with known level of immune response indicated that alpha and omega gliadins both originating from the D genome contain most of the epitopes (Juhász et al., 2018). These alpha gliadins, in general, contain all the composing epitopes (DQ2.5-glia- $\alpha$ 1a, DQ2.5-glia- $\alpha$ 1b, DQ2.5-glia- $\alpha$ 2) of the alpha 33-mer in a different number and combination. Using trypsin as protease in the nano LC-MS/MS analysis we have identified seven alpha gliadins in MVGB787, six alpha gliadins in MVGB770 while only one–one alpha gliadin has been identified in the MVGB1177 and MVGB748 genotypes. While proteins identified from the MVGB787 and MVGB770 genotypes contain the DQ2.5-glia- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 3 epitopes these were absent in the proteins identified from the two low immune responsive genotypes. Additionally, neither DQ2.5-glia- $\alpha$ 1b nor DQ2.5-glia- $\alpha$ 2 epitopes were present in the alpha gliadin sequences detected in the four genotypes. Ráki and co-workers (2017) proved that many T cell clones (TCCs) derived from adults and children with celiac disease recognized peptides from  $\omega$ - and  $\gamma$ -gliadin, while only about 10% of the clones were found to be reactive against  $\alpha$ -gliadin peptides (all these lines responded to 33mer peptide). This indicated that the immuno-dominance of  $\alpha$ -gliadin epitopes in T cell lines (TCL) is not correlated with the presence of these  $\alpha$ -gliadin reactive clones in TCCs. Furthermore, they found that cross reactivity is frequent, especially in TCCs recognizing peptides representing DQ2.5-glia- $\omega$ 1, DQ2.5-glia- $\omega$ 2 and DQ2.5-glia- $\gamma$ 4 epitopes, while some TCCs recognize peptides from  $\gamma$ -gliadin without cross-reactivity. TCCs monospecific for the DQ2.5-glia- $\gamma$ 4c and DQ2.5-glia- $\gamma$ 4b epitopes also exist. The gamma gliadins have a key role in the early stage of disease development, contrary to the earlier view that alpha gliadins would have significant dominance for triggering celiac disease (Ráki et al., 2017; Diós et al., 2021).

Consequently, einkorn as diploid wheat species has fewer immune reactive sequences, causing reduced immunogenicity, associated with quite high gluten content. However, our analyses with the purified DG4 antibody indicated that the abundant cross-reactivity is related to the main celiac gamma epitope, QPQQPF.

Low immune response against the storage proteins of the selected *Triticum monococcum* L. ssp. *monococcum* genotypes indicate their potential use for low-gluten nutritional studies, base material testing and further food processing. Based on our results four einkorn genotypes (MVGB770, MVGB787, MVGB748 and MVGB1177) showed particularly low immune reactive values. However, further investigations including clinical studies are required to confirm the low immune response of these einkorn genotypes and to understand the stability of the low immune reactive levels in different environmental conditions. According to the new dietary trends these einkorn genotypes could be incorporated into the health supporting diets and the higher glutenin/gliadin ratio can provide better baking quality compared to einkorns with low gluten content.

## 5. Conclusion

Following the extensive screening of a Gene Bank collection of 208 genotypes with serological ELISA four *Triticum monococcum* L. ssp. *monococcum* genotypes have been selected for detailed proteomics and immunogenic analyses due to their significantly lower immune reactivity.

Based on the R5 ELISA results the R5 reactive protein content was

below 100 ppm in all four einkorn genotypes; however the SE- and RP-HPLC results show comparable gluten values to bread wheat, and thus indicate not the low gluten content but a different gluten protein composition, with prolamins sequences depleted in known immune responsive regions. The nanoLC-MS/MS analyses highlighted, that the gamma gliadins of einkorns have a major role in celiac disease. Besides, non-prolamins storage proteins were also identified with immune reactivity in einkorns. The low immune response characteristic for these genotypes can be utilized in breeding programs targeting wheat production with a modified gluten content and composition.

Our results indicate the benefits of using high-throughput large scale pre-screening for genotype selection, and a complex examination platform of the best candidates to get relevant information about the genetic diversity of celiac disease responsive proteins in the analyzed einkorn genotypes.

## CRedit authorship contribution statement

**Zsófia Birinyi:** Investigation, Writing - original draft, Visualization, Formal analysis. **Dalma Réder:** Investigation. **Ádám Diós:** Investigation, Formal analysis. **Ilma R. Korponay-Szabó:** Writing - review & editing, Supervision. **Éva Hunyadi-Gulyás:** Investigation, Formal analysis. **Christakis George Florides:** Investigation, Formal analysis. **Angéla Juhász:** Formal analysis, Writing - review & editing, Investigation, Visualization, Supervision. **Gyöngyvér Gell:** Conceptualization, Investigation, Formal analysis, Writing - review & editing, Supervision, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Ethical Statement

Informed written consent to the collection of serum samples was given by the parents. All the methods of subject recruitment, data collection, and experiments were performed in accordance with relevant guidelines and regulations. All experiments were approved by the ethics committee of the Heim Pál National Paediatric Institute, Budapest and the Semmelweis University Regional and Institutional Committee and Research Ethics. Serum samples were collected during the diagnostic procedure of celiac disease for the clinical investigation of celiac antibodies according to ESPGHAN guidelines and used later for the experiments with the permission of the Ethical Committee of the Heim Pál National Paediatric Institute, Budapest, Hungary.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.131148>.

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