Food Chemistry 264 (2018) 101–107

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Detection and quantitation of immunogenic epitopes related to celiac disease in historical and modern hard red spring wheat cultivars

Maneka Malalgoda[a], Steven W. Meinhardt[b], Senay Simsek[a,*]

[a] North Dakota State University, Department of Plant Sciences, NDSU Dept. 7670, PO Box 6050, Fargo, ND 58108-6050, USA
[b] North Dakota State University, Department of Plant Pathology, NDSU Dept. 7660, PO Box 6050, Fargo, ND 58108-6050, USA

ARTICLE INFO

Keywords:
Celiac disease
Immunogenic epitopes
Wheat
α-Gliadin
Proteomics

ABSTRACT

Celiac disease (CD) develops in genetically susceptible individuals as a result of ingesting gluten-forming proteins found in cereals, such as wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.) and barley (*Hordeum sativum* L.). There are claims that breeding practices have changed wheat protein chemistry over the years and this has resulted in modern wheat being more antigenic in terms of CD as opposed to historical wheat. The aim of this study was to detect and quantify celiac-disease-initiating peptides of α-gliadin proteins in historical and modern spring wheat cultivars. The results indicate that immunogenic epitopes are detected in both historical and modern spring wheat cultivars irrespective of release year. Quantitation indicated that the amount of immunogenic epitopes glia-α9 (PFPQPQLPY) and glia-α20 (FRPQQPYPQ), and total α-gliadin varied randomly across the cultivars that were analyzed, suggesting there is no association between cultivar release year and amounts of immunogenic epitopes and α-gliadin.

1. Introduction

Celiac disease is a genetically predisposed autoimmune disease, which is one of the most common food intolerances in the world (Wieser & Koehler, 2008). It is defined as an inflammatory disease, which causes damage to the upper small intestine. Celiac disease is most prevalent in Europeans and those with European ancestry (Gallagher, Gormley, & Arendt, 2004). In the Unites States, it is estimated that 1 in 111 people suffer from CD, which accounts for approximately 1% of the general population. In celiac disease, inflammation is caused by the ingestion of cereals, such as wheat, barley, rye and, possibly, oat products (Wieser & Koehler, 2008). The key factors, which cause the immune reaction, are the gluten-forming proteins and homologous proteins found in some cereals.

Gluten-forming proteins are composed of gliadin and glutenin proteins. These proteins act as antigens in genetically susceptible individuals and trigger an immune response. The peptides, which illicit the immune reaction, are known as immunogenic epitopes/ T-cell epitopes/ antigenic peptides. Gliadins and glutenins are involved in forming the gluten network, which is responsible for many of the characteristics of breads and pastas, during mixing of dough. Gliadins are divided into three subgroups, namely, α-/β-, γ- and ω- and range in molecular weight from 28 to 55 kDa (Bonomi, Iametti, Mamone, & Ferranti, 2013). Studies have shown that the alcohol soluble prolamin (proline and glutamine) fraction consisting of gliadins is the most antigenic component in wheat proteins (Wieser & Koehler, 2008).

One of the main purposes of wheat breeding is the introduction of favorable traits, such as high yield, disease resistance and drought tolerance. In North Dakota, many improved wheat cultivars have been released during the last 100 years. There is ongoing debate about the antigenicity of modern wheat in comparison to historical wheat, and whether breeding practices contributed towards increasing CD antigenicity of modern wheat. In this context, van den Broeck, Cordewener, Nessen, America, and van der Meer (2015) quantified two immunogenic epitopes, glia-α9 (PFPQPQLPY) and glia-α20 (FRPQQPYPQ), and total α-gliadin in three wheat cultivars. The results showed that a modern hexaploid wheat cultivar, Toronto, contained a higher amount of the immunogenic peptides in comparison to an older hexaploid wheat cultivar, Minaret, and a tetraploid landrace, Dibillik. Additionally, van den Broeck et al., 2010 used monoclonal antibodies against the same CD epitopes to determine differences in antigenicity between 36 modern European wheat cultivars and 50 landraces (representing wheat varieties from a century ago), and found more glia-α9 peptide in modern wheat than landraces, with the exception of a few modern cultivars.

Prandi, Mantovani, Galaverna, and Sforza (2014) conducted a study to determine celiac disease antigenicity of durum wheat cultivars using an isotopically labelled internal standard for the immunogenic epitope

* Corresponding author.
E-mail address: senay.simsek@ndsu.edu (S. Simsek).

https://doi.org/10.1016/j.foodchem.2018.04.131
Received 7 July 2017; Received in revised form 20 February 2018; Accepted 28 April 2018
Available online 30 April 2018
0308-8146/

glia-α9. The results indicated that there is considerable variability in the amount of immunogenic peptides among the wheat cultivars that were investigated, and that environment has a very low effect in comparison to genetic factors. In a similar study done by the same group, the CD antigenicity of *Triticum durum* and *Triticum aestivum* were compared. It was found that the content of immunogenic epitopes have positive correlations with total protein content, and the amount of gliadin. Additionally, the amount of immunogenic peptides in *Triticum durum* was found to be lower than that of *Triticum aestivum*, and high variability was observed in the different durum samples that were analyzed.

Additionally, Prandi, Tedeschi, Folloni, Galaverna, and Sforza (2017) studied the antigenicity of old and modern wheat, and determined that older cultivars have higher amounts of immunogenic peptides compared to modern cultivars, thus, concluding that old varieties are unsafe for consumption by CD patients. Gregorini, Colomba, Ellis, and Ciclitira (2009) also detected CD epitopes in the ancient varieties investigated, and found higher levels of α-gliadin in these samples, indicating that ancient wheat is unsafe for CD patients. Gianfrani et al. (2012) evaluated the CD antigenicity of two diploid wheat cultivars of *Triticum monococcum* ssp. *monococcum*, and found that the two diploid wheat cultivars were antigenic to CD patients; however, one of the varieties was concluded to be less antigenic than the other, since it did not trigger the innate immune response. Similar observations were made by Colomba and Gregorini (2012).

In the same manner, intestinal biopsies from CD patients have been used to assess the antigenicity of ancient and modern wheat. Suligoj, Gregorini, Colomba, Ellis, and Ciclitira (2013) found that wheat varieties trigger the T-cell response associated with CD irrespective of their origin (ancient/ modern) or ploidy. Additionally, they found that response to these peptides is heterogeneous among CD patients. Thus, bringing another complicated aspect to this study, as each CD patient has a unique response when immunogenic epitopes are encountered. Therefore, to fully understand if a certain wheat variety is antigenic, multiple T-cell lines from different CD patients should be used.

The objective of this study was to determine changes in the different gliadin protein components, and identify and quantify CD causing α-gliadin epitopes and total α-gliadin, in historical and modern wheat cultivars grown in the state of North Dakota, during the last 110 years, and thereby, compare the antigenicity of the different wheat cultivars. In this study, historical wheat refers to cultivars released prior to 1966, and modern wheat refers to cultivars released after 1966. Antigenicity, in this context, refers to the amount of immunogenic epitopes and total α-gliadin.

2. Materials and methods

2.1. Materials

Thirty wheat cultivars released in North Dakota from 1910 to 2013 were used in this study. The cultivars and their release years (in parenthesis) are as follows: Marquis (1910), Ceres (1926), Pilot (1939), Rival (1939), Vesta (1942), Mida (1944), Conley (1955), Justin (1963), Fortuna (1966), Waldron (1969), Olaf (1972), Butte (1977), Len (1979), Stoa (1984), Butte86 (1986), Grandin (1989), Pioneer 2375 (1990), Gunner (1995), Russ (1995), Reeder (1999), Alsen (2000), Granite (2002), Freyer (2004), Steele-ND (2004), Glenn (2005), Faller (2007), RB07 (2007), Barlow (2009), Velva (2012), and Elgin (2013). Seeds for this study were obtained from the North Dakota State University Hard Red Spring Wheat Breeding Program. The cultivars were grown in Casselton, ND (US) in 2013 in approximately 1 m × 45 m strip plots. The samples were planted during the first week of June, and harvested during the first week of September. Once harvested, the samples were cleaned and milled at an in-house facility at the North Dakota State University. A Buhler ML-202 laboratory scale mill was used to mill the samples, and subsequently used in the experiments described below.

2.2. Reverse-phase HPLC (RP-HPLC) analysis of gliadin proteins

Gliadin proteins were extracted and separated by RP-HPLC according to the method of Lookhart and Bean (1995), with modifications. A volume of 750 µl of 70% ethanol was added to 250 mg of flour and shaken at 1400 rpm for 1 h at 30 °C in a heating block (Eppendorf Thermomixer R, Eppendorf AG, Hamburg, Germany). The mixture was then centrifuged at 4550*g* for 10 min (Eppendorf Centrifuge 5415C, Eppendorf AG, Hamburg, Germany), and filtered through a 0.45 µm nylon filter (VWR International, Radnor, U.S.A.). The Pierce™ BCA assay kit (Thermo Fisher Scientific, Waltham, U.S.A.) was used to measure the protein content in each extract. After the protein content was determined, each extract was diluted to 13 µg/µl using water filtered through a 0.45 µm nylon membrane filter. The samples were then heat-treated at 80 °C for 3 min in a heating block prior to running through the HPLC.

An Agilent Zorbax 300SB-C18 (4.6 × 250 mm, 5 µm) (Agilent Technologies, Waldbroann, Germany) column was used for separation of the protein extracts. Two solvents, A and B, were used in the analysis (Solvent A: 95% water (v/v), 5% acetonitrile (ACN; v/v) and 0.1% trifluoroacetic acid (TFA; v/v), and solvent B: 100% acetonitrile with 0.08% (v/v) TFA). The absorbance of the two solvents were adjusted with TFA so that both solvents would have similar absorbance at 210 nm. Subsequently the solvents were filtered through a 0.45 µm nylon membrane. Gliadins were eluted in a linear multistep gradient at 65 °C at a flow rate of 1 ml/min. The injection volume was 23 µl (approximately 300 µg of protein based on the BCA assay). The signal was detected at 210 nm using an Agilent 1100 variable wavelength detector (Agilent Technologies, Waldbroann, Germany). The elution gradient started at 21% solvent B, which was maintained for the first 5 min. From 5 to 10 min, the percentage of B was increased from 21% to 26%. Between 10 and 60 min, the percentage of B was increased to 50%, which was maintained until 63 min. From 63 to 64 min solvent B was increased to 100%. From 64 to 66 min, the gradient of B was brought down to 21%. A post time of 15 min was added after each run.

The HPLC data was processed using an in-house program coded using MATLAB (2015, The MathWorks, Natick, U.S.A.). UV absorbance values were interpolated to 0.005 min intervals by the 'spline' function in MATLAB. Absorbance area values were calculated for specific peaks using the interpolated absorbance data. The SAS software (Version 9.3, SAS Institute, Cary, U.S.A.) was used for the statistical analysis of the results, and correlation analysis was done using the 'CORR' procedure in SAS.

2.3. Gliadin extraction and digestion for liquid chromatography-mass spectrometry (LC-MS)

Gliadin proteins were extracted as previously mentioned, and the amount of protein in each extract was quantified using a Pierce™ BCA assay kit (Thermo Fisher Scientific, Waltham, U.S.A.). Afterwards, each sample was mixed with SDS-PAGE sample buffer so that each sample would contain 40 µg of protein. Afterwards, the samples were boiled for 5 min. An 8% Tris-Tricine gel was used to run the samples (Schägger & von Jagow, 1987). The gel was run for 15 min at 55 V so that the proteins would be incorporated in the gel. For staining the gels, a protocol adapted from the European Molecular Biology Laboratory (EMBL) was used. The gel was washed briefly with water, then fixed in a solution containing 45% methanol (v/v), 45% water (v/v) and 10% acetic acid (v/v) for 15 min. Afterwards, the gel was washed again with water and fixed in the same solution for another 15 min, after which, the gel was washed again. The gel was stained in Colloidal Coomassie blue overnight. The following day, the individual gel bands were cut using a sterile razor blade and stored at −20 °C in 1.5 ml Eppendorf tubes for mass spectrometric analysis. The gliadin gel bands were

subjected to in-gel digestion with chymotrypsin according to the method of Shevchenko, Wilm, Vorm, and Mann (1996). The digested samples were stored at −80 °C until mass spectrometric analysis was performed. Prior to analysis, the samples were desalted using the STAGE (Stop And Go Extraction) tip desalting procedure according to Rappsilber, Ishihama, and Mann (2003).

2.4. Qualitative LC-MS and data analysis

Approximately 1.5 µg of the chymotrypsin digested peptide mixtures were used for LC-MS analysis on an Orbitrap Velos system (Thermo Fisher Scientific, Waltham, U.S.A.) as previously described by Lin-Moshier, et al. (2013). Identical LC conditions were used and the following modifications were made to the MS acquisition settings: the MS1 scan range was 360–1800 *m/z*; the minimum abundance for MS/MS trigger was 10,000 counts; lock mass was not selected; dynamic exclusion settings were: list size 200 values, duration 30 s, exclusion mass tolerance ± 10 ppm.

The PEAKS 7.0 software (Bioinformatics Solutions, Waterloo, Canada) was used to analyze the .RAW data files against the database nr_triticinae and a database of common contaminants 1648030_20150930_cRAP123 downloaded from NCBI. Search parameters were as follows: enzyme: chymotrypsin; max missed cleavages: 2; non-specific cleavages: 2; fixed modifications: carbamidomethylation; variable modifications: oxidation; max variable PTM per peptide: 3; parent mass error tolerance: 50 ppm, fragment mass error tolerance: 0.1 Da. Results filter parameters were as follows, peptide (-l0lgP): ≥15; protein (-l0lgP): ≥20. False discovery rate for peptides and proteins was 0.8–19.5 and 0.0–5.5 respectively. The data was used to determine if immunogenic epitopes can be detected in the historical and modern wheat cultivars being investigated. Additionally, a binary dataset was created according to the presence or absence of immunogenic epitopes and this information was used to produce a dendrogram according to Ward's method using the SAS software (Version 9.3, SAS Institute, Cary, U.S.A.). The cubic clustering criterion, pseudo F statistic and the pseudo t^2 statistic were used to determine the number of clusters in the dendrogram.

2.5. Relative quantification of immunogenic epitopes

The peptides proposed by van den Broeck et al. (2015) were used in the quantification of immunogenic epitopes glia-α9 (PFPQPQLPY), glia-α20 (FRPQQPYPQ), and the amount of total α-gliadin. Peptides P1-P5, P6-P7 and P8-P9 were used for the quantification of glia-α9, glia-α20 and total α-gliadin respectively. These sequences were imported into the Skyline software to produce mass lists (MacLean et al., 2010). These mass lists were imported into the Analyst software (Sciex, Framingham, U.S.A.) and used to generate unscheduled SRM methods on a Qtrap 5500 (Sciex, Framingham, U.S.A.) to optimize the LC gradient and select optimal transitions (mass lists with collision energy are presented in Supplementary Table 1). The final optimized transitions were built into a scheduled method, with parameters described in Supplementary Table 2. The QTRAP 5500 was equipped with an Agilent 1100 capillary LC system. Solvents A and B were 98% water (v/v), 2% ACN (v/v)and 0.1% formic acid (v/v), and ACN and 0.1% formic acid (v/v) respectively. A three-step gradient was used to separate the peptides. The gradient started with 0% B, which was increased to 5% B in the next minute. From min 1–17.5 the percentage of B was increased to 45%, and then from 17.5 to 18 min it was increased to 85%, which was held until min 21. From 21 to 21.5 min, the percentage of B was reduced to 0, which was held till 24 min. A flow rate of 8 µl/min was used from 0 to 19.99 min, which was changed to 10 µl/min from 20 to 24 min. The column was 100 × 0.3 mm, with 2.7 µm HALO C18 particles with 90 Å pore size (Eksigent, Redwood city, U.S.A.) and was heated to 55 °C using a sleeve column heater (Analytical Sales & Services, Flanders, U.S.A.). The digested samples were reconstituted to 2 µg/µl, and mixed with an equal volume of heavy-labeled standard peptides at various concentrations matching the endogenous signal of a pooled sample. SRM data was imported into Skyline for quantitation by normalizing endogenous signals to that of the spiked heavy peptides.

2.6. Absolute quantification of immunogenic epitopes

The absolute quantification of peptides was performed using calibration curves. The amount of the immunogenic epitopes glia-α9 and glia-α20, and total α-gliadin were quantified in 10 samples, across the cultivars of 100 years that were investigated. Heavy labelled peptides P1-P9 were diluted to 0.1 nmol/µl solutions. The dilution solvent comprised of 98% water (v/v), 2% ACN (v/v) and 0.1% formic acid (v/v). These solute ions were then diluted to give solutions of the following concentrations (fmol/µl): 5, 10, 20, 50, 70, 100, 200, 500, 700, 1000, 2000, 5000 and 10000. To construct the 'blank standard curve', a solvent composed of 98% water, 2% ACN and 0.1% formic acid was used. A 1:1 dilution of each of the heavy labelled peptide concentrations was done with the solvent, and 2 µl of the resulting mixture was injected to the QTRAP 5500 instrument. The conditions, the solvents and the gradient used were same as explained in the previous section. To construct the 'matrix standard curve', the 1:1 dilution was done with a wheat sample that was reconstituted to 2 µg/µl. All injections were done in duplicate during this experiment. The area for the heavy ions in the 'blank standard curve runs' and the 'matrix standard curve runs' were plotted against each other to determine the matrix effect. When conducting this analysis, the sum of the highest-ranking transitions was used as a measure of the area of each ion. Calibration curves created for the heavy transitions in 'matrix standard curve' runs were used in the quantification process.

3. Results and discussion

3.1. Reverse-phase-HPLC analysis of gliadin proteins

The peaks identified in the RP-HPLC chromatogram were analyzed for correlations with release year, to determine which gliadin fractions changed in amount in the last 100 years. The regions of the chromatogram corresponding to the different gliadin types, shown in Supplementary Fig. 1, were identified according to previous studies (Gessendorfer, Koehler, & Wieser, 2009; Piston, Gil-Humanes, Rodriguez-Quijano, & Barro, 2011; Wang et al., 2012). Supplementary Table 3 shows the correlations between the different gliadin types and release year. The results of the correlation study indicate that only the RP-HPLC chromatogram area corresponding to ω-gliadin proteins showed significant positive correlations ($P \leq 0.05$) with release year. Thus, the amount of ω-gliadin proteins is higher in modern wheat in comparison to historical wheat. The α-/β- and γ-gliadin proteins did not show significant correlations ($P \leq 0.05$) with year, therefore, the

Table 1
Correlations between area of ω- and γ-gliadin peaks with release year.

Gliadin type	Peak	Retention time (min)	Mean	Min	Max	SD	Correlation with year
ω-Gliadin	1	12.1–13.0	137.8	39.8	297.3	61.3	0.472**
	2	13.2–14.1	137.3	25.7	341.6	81.2	0.633***
	3	14.6–15.2	16.3	7.2	50.6	12.8	0.491**
	4	18.2–18.7	13.1	8.3	34.3	7.1	−0.649***
	5	20.1–21.0	225.5	29.7	470.7	130.9	−0.466**
	6	21.7–22.2	47.8	6.2	130.5	39.9	0.672***
γ-Gliadin	7	40.2–40.8	327.5	217.6	504.1	56.7	0.474**
	8	42.9–43.4	68.8	7.6	144.1	32.2	0.578***
	9	50.0–50.6	198.0	5.2	579.6	200.9	−0.545**
	10	51.8–52.3	31.1	2.9	80.6	22.6	0.714***

** = $P \leq 0.01$.
*** = $P \leq 0.001$.

Table 2
Immunogenic epitopes detected in historical and modern spring wheat cultivars.

Cultivar	Year	α/β-gliadin					γ-gliadin								ω-gliadin	
		FPQQPYP-Q	PFPQPQL-PY	PYPQPQL-PY	PQPQLPY-PQ	QGSFQPS-QQ	PQQSFP-QQQ	IQPQQPA-QL	QQPQQP-YPQ	SQPQQQ-FPQ	PQPQQQ-FPQ	QQPQQP-FPQ	PQPQQP-FCQ	QQPFPQ-QPQ	PFPQPQ-QPF	PQP-QQP-FPW
Marquis	1910	✓	✓	✓	✓	✓	✓	✓			✓	✓		✓		
Ceres	1926	✓	✓		✓	✓	✓	✓						✓		
Rival	1939	✓	✓		✓	✓	✓	✓				✓		✓	✓	
Pilot	1939	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓		✓		
Vesta	1942	✓	✓	✓	✓		✓	✓	✓		✓	✓		✓	✓	✓
Mida	1944	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓		✓		
Conley	1955	✓	✓		✓	✓	✓	✓						✓	✓	
Justin	1963	✓	✓	✓	✓	✓	✓	✓			✓	✓		✓	✓	✓
Fortuna	1966	✓	✓			✓	✓	✓						✓	✓	
Waldron	1969	✓	✓	✓	✓		✓	✓				✓		✓	✓	
Olaf	1972	✓	✓		✓	✓	✓	✓						✓	✓	
Butte	1977	✓	✓	✓	✓	✓	✓	✓				✓		✓	✓	✓
Len	1979	✓	✓	✓	✓	✓	✓	✓			✓	✓		✓	✓	
Stoa	1984	✓	✓		✓	✓	✓	✓				✓		✓	✓	
Butte-86	1986	✓	✓	✓	✓	✓	✓	✓	✓			✓		✓	✓	✓
Grandin	1989	✓	✓	✓	✓	✓	✓	✓				✓		✓	✓	✓
Pioneer-237-5	1990	✓	✓	✓	✓	✓	✓	✓				✓		✓	✓	✓
Gunner	1995	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓		✓	✓	✓
Russ	1995	✓	✓		✓	✓	✓	✓						✓	✓	
Reeder	1999	✓	✓	✓	✓		✓	✓				✓		✓	✓	
Alsen	2000	✓		✓	✓	✓	✓	✓				✓		✓	✓	✓
Granite	2002	✓	✓	✓	✓		✓	✓				✓		✓	✓	
Freyr	2004	✓	✓	✓	✓	✓	✓	✓				✓		✓	✓	✓
Steele-Nd	2004	✓	✓	✓		✓	✓	✓				✓		✓		
Glenn	2005	✓	✓		✓	✓	✓	✓				✓				
Faller	2007	✓	✓	✓	✓	✓	✓	✓	✓			✓		✓	✓	
Rb07	2007	✓	✓		✓	✓	✓	✓						✓	✓	
Barlow	2009	✓	✓	✓	✓	✓	✓	✓	✓			✓		✓	✓	✓
Velva	2012	✓	✓	✓	✓	✓	✓	✓			✓	✓		✓	✓	
Elgin	2013	✓	✓		✓	✓	✓	✓						✓	✓	

amount of these proteins in historical and modern wheat is not significantly different. Several individual peaks in the ω-gliadin and γ-gliadin regions of the chromatogram showed significant correlations ($P \leq 0.01$) with release year (six ω-gliadin peaks and four γ-gliadin peaks). The peaks that were correlated with release year are shown in Supplementary Fig. 2, and the quantitative values are shown in Table 1. Individual peaks in the α/β-gliadins region did not significant correlations with year. Overall, the HPLC results indicate that ω-gliadin proteins and few γ-gliadin peaks increased with release year. However, a correlation was not found between the amount of α-gliadin proteins and year. Since previous studies link the antigenicity of wheat with the amount of α-gliadin, this is an indication that modern wheat may not contain higher amounts of immunogenic epitopes.

3.2. Qualitative analysis of immunogenic epitopes

Following mass spectrometric analysis of the samples, the PEAKS software was used for the sorting and exporting of the data. The LC-MS results were analyzed to determine the presence of immunogenic epitopes causing celiac disease (Table 2). The antigenic epitopes identified by Sollid, Qiao, Anderson, Gianfrani, and Koning (2012) were used in this section. Table 2 shows that immunogenic epitopes, which trigger the immune reaction related to CD are found in both historical and modern spring wheat cultivars. All five immunogenic epitopes belonging to the α/β-gliadin proteins were found in majority of the cultivars. It is interesting to note that the epitope PFPQPQLPY was detected in all cultivars except cultivar Alsen released in 2000. Two epitopes (SQPQQQFPQ and PQPQQPFCQ) found in the γ-gliadin proteins were not detected in any cultivar. Of the two ω-gliadin peptides, one (PFPQPQQPF) was found in almost all cultivars, while the second (PQPQQPFPW) was found in one third of the cultivars. These results show that immunogenic epitopes are present in both historical and modern spring wheat cultivars irrespective of release year. To analyze the relationship between the presence of these epitopes and release year, cluster analysis was performed according to Ward's method (Ward, 1963).

At cutting point 2.7, the cluster analysis produced 5 clusters (Fig. 1). The release year of the cultivars in the different clusters is as follows, cluster A: 1942–2009, B: 1969–2002, C: 1910–2012, D: 1926–2013, E: 1939–2005. As such, in this dendrogram the cultivars did not cluster according to release year. Thus, the cultivars in any of the clusters formed do not represent only modern or historical wheat cultivars. The ANOVA test showed that there is no significant difference ($P \geq 0.05$) between clusters for release year. Thus, the results suggest that the presence or absence of immunogenic epitopes is independent of release year, indicating that historical and modern wheat may not be different in terms of their CD antigenicity.

The ANOVA test showed significant differences ($P \geq 0.05$) between clusters with regard to the area of peak 7 (γ-gliadin peak-Supplementary Fig. 2). That is, cluster B was significantly different than the other clusters. The α/β-gliadin immunogenic epitope QGSFQPSQQ was not detected in four of the thirty cultivars that were analyzed, and three of these cultivars are present in cluster B. This could be the cause for the significant difference that was observed.

Our results suggest that there may not be a difference in antigenicity between historical and modern wheat cultivars, since immunogenic epitopes were detected in both historical and modern cultivars, irrespective of release year. The results of the cluster analysis further support this view. That is, in terms of the presence of immunogenic epitopes, modern wheat is not higher in antigenicity in comparison to historical wheat. Some immunogenic epitopes may not have been detected due to limitations in the instrument or the method used for the analysis, and non-detection of a peptide does not imply that the peptide is absent in the gliadin extract. Although the qualitative analysis gives some indication about CD antigenicity of historical and modern wheat, quantitative analysis was needed to determine if changes in the amount

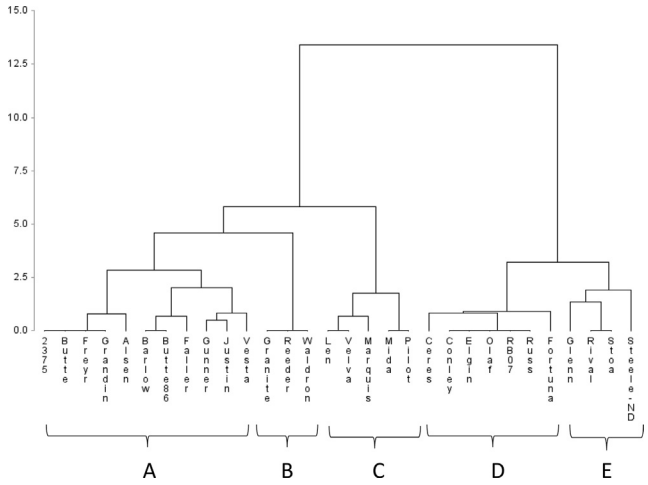

Fig. 1. Dendrogram based on the presence or absence of immunogenic epitopes in the cultivars analyzed. This dendrogram was produced using Ward's clustering method referenced in the methods section. Five clusters (A-E) were identified at cutting point 2.7, which grouped cultivars randomly. That is, historical or modern cultivars were not grouped in specific clusters, which is an indication that the celiac antigenicity of wheat is not related to the release year of cultivar. According to the dendrogram, the release years of the cultivars in each cluster range a time span of at least 33 years.

of CD antigenic epitopes occurred over the last century. In mass spectrometric analysis of proteins, the choice of protease contributes towards determining which peptides are detected. In cereal gliadins, trypsin does not produce peptides with appropriate mass for detection through mass spectrometry. This is because cereal gliadins are low in lysine and arginine, thus trypsin has limited cleavage sites. Chymotrypsin cleavage sites are C-terminal to aromatic amino acids, if the next amino acid is not proline. Gliadins contain numerous aromatic amino acids which make chymotrypsin the appropriate protease for their digestion (Salplachta, Marchetti, Chmelik, & Allmaier, 2005).

3.3. Quantitation of immunogenic epitopes

In the relative quantification of immunogenic epitopes, the ratio between the isotopically labeled and native peptides was considered as a measure of the amount of the epitopes present in the wheat sample. Table 3 shows the ratios that were calculated. Cultivar Steele-ND released in 2004, has a relatively high amount of immunogenic epitopes compared to the other cultivars that were analyzed. In the same manner, cultivar Pilot released in 1939 has high amounts of the epitope FRPQQPYPQ and total α-gliadin. Cultivar Russ released in 1995, is of lower antigenicity in comparison to the others, since the ratios are low for this cultivar. Overall, the relative amounts of immunogenic epitopes glia-α9 (PFPQPQLPY), glia-α20 (FRPQQPYPQ) and total α-gliadin range from 1.8–17.8, 0.05–9.6 and 0.05–18.5 respectively in the cultivars investigated. The ratio determined for the oldest and newest cultivars were 9.8 and 5.3 for epitope glia-α9, and 3.3 and 0.2 for epitope glia-α20.

As explained in the experimental procedure, in order to determine the matrix effect caused by the wheat samples, the area of the transitions for each heavy labeled peptide in the blank and matrix standard curve runs were plotted against each other (Supplementary Fig. 3-A). If the slope was not significantly different than 1, it was assumed that there was no matrix effect, and that the standard curves created were applicable for the quantification of any of the thirty wheat samples. The peptides P1-P5, and P8-P9, the gradient ranged from 0.92 to 1.2. Calibration curves in matrix were used for the quantification of the peptides. Supplementary Fig. 3B shows an example of such a curve. Information about standard curves are presented in Supplementary

Table 3
Relative quantification of immunogenic epitopes.

Cultivar	Year	PFPQPQLPY[1]	FRPQQPYPQ[2]	Total α-gliadin[3]
Marquis	1910	9.8	3.3	9.5
Ceres	1926	4.1	0.2	0.2
Pilot	1939	12.4	5.3	9.2
Rival	1939	3.5	0.1	0.05
Vesta	1942	9.3	4.4	5.4
Mida	1944	9.0	3.6	6.9
Conley	1955	2.3	0.05	0.04
Justin	1963	10.4	3.1	9.3
Fortuna	1966	2.8	0.1	0.07
Waldron	1969	7.6	1.7	3.8
Olaf	1972	2.7	0.1	0.1
Butte	1977	9.7	3.5	8.7
Len	1979	8.6	2.7	7.8
Stoa	1984	2.8	0.1	0.07
Butte86	1986	10.9	5.1	12.6
Grandin	1989	12.2	5.1	12.7
Pioneer-2375	1990	13.7	3.6	9.3
Gunner	1995	11.0	3.2	5.5
Russ	1995	1.8	0.03	0.01
Reeder	1999	6.4	2.9	6.2
Alsen	2000	7.8	2.2	6.9
Granite	2002	8.3	2.7	4.9
Freyr	2004	9.3	2.1	4.6
Steele-Nd	2004	17.8	9.6	18.5
Glenn	2005	6.8	0.2	0.4
Faller	2007	6.0	2.4	3.7
Rb07	2007	2.3	0.1	0.05
Barlow	2009	11.0	3.2	5.9
Velva	2012	6.5	1.3	3.6
Elgin	2013	5.2	0.2	0.12

[1] The peptide PFPQPQLPY is the sum of *P*1-5 and represents the glia-α9 epitope.
[2] The peptide FRPQQPYPQ is the sum of *P*6-7 and represents the glia-α20 epitope.
[3] Total a-gliadin was calculated from the sum of P8 and P9.

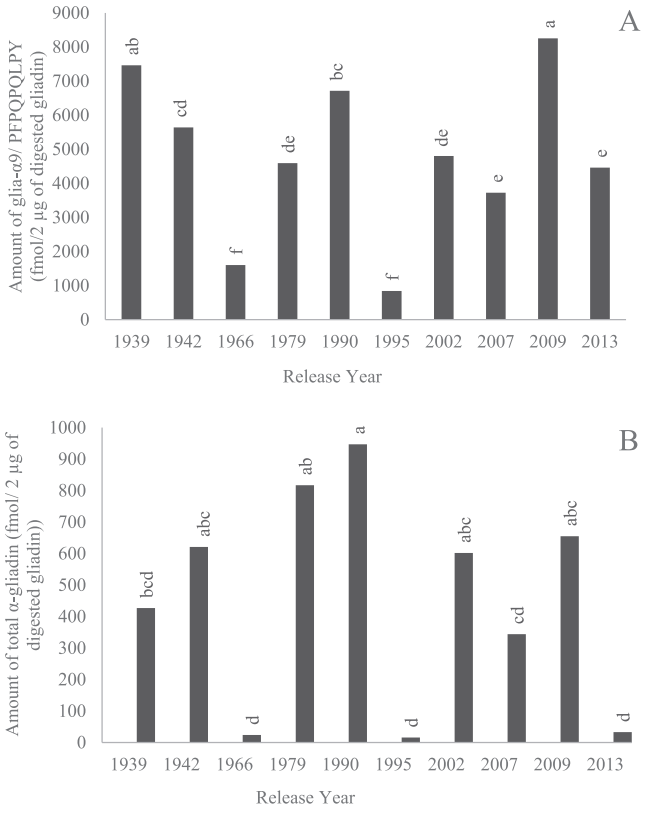

Fig. 2. Graph showing the amount of immunogenic epitope glia-α9 (PFPQPQLPY) (A) and total α–gliadin (B). Columns with the same letter in the same graph are not significantly different at the 5% level. The graph shows the amount of the glia-α9 and total α-gliadin varied randomly across the years that were analyzed, indicating that there is no relation between cultivar release year and CD antigenicity.

Table 4.

For the absolute quantification of immunogenic epitopes, ten cultivars were randomly chosen to span the hundred years that were being analyzed. A shift in the retention time of P6 and P7 was observed in some runs, thus accurate calibration curves for these peptides could only be obtained in the range from 5 to 50 fmol. Due to this issue, we were unable to quantify the immunogenic epitope glia-α20 (FRPQQPYPQ) for most cultivars analyzed. Cultivar Steele-ND was not used in this experiment as the high amount of peptides was expected to saturate the column.

The amount of immunogenic epitope was determined in fmol per 2 µg of chymotrypsin digested gliadin. The values determined for P1-P9 are shown in Table 4. For P1-P5, which were used to quantitate immunogenic epitope glia-α9 (PFPQPQLPY), the amount ranged from ≤5 (Russ, P3) to 4168 (Barlow, P5). Peptides P8 and P9, which were used

Table 4
Quantification of immunogenic epitopes using calibration curves[1].

Cultivar	Year	Amount of peptide (fmol/ 2 µg of chymotrypsin digested gliadin)								
		P1	P2	P3	P4	P5	P6	P7	P8	P9
Pilot	1939	1861.9	911.5	100.3	567.5	4024.1	≥50	≥50	337.7	88.9
Vesta	1942	1528.3	1014.1	125.7	478.2	1724.7	≥50	≥50	523.1	75.3
Fortuna	1966	895.3	19.6	134.5	143.3	385.5	≥50	32.3	7.9	15.6
Len	1979	1692.3	218.8	271.2	651.5	1758.6	≥50	32.9	726.6	90.8
Pioneer-2375	1990	1579.7	1132.3	1335.3	740.8	1929.2	≥50	≤5	823.5	123.3
Russ	1995	364.5	101.3	≤ 5	49.0	306.6	5.7	≤5	2.5	13.6
Granite	2002	1616.7	27.8	1091.3	643.5	1425.0	≥50	≥50	518.3	83.8
Faller	2007	1530.6	302.1	176.4	572.2	1144.7	≤5	6.4	290.8	53.2
Barlow	2009	1627.4	1159.5	355.4	933.1	4178.5	≥50	37.3	559.4	96.0
Elgin	2013	1200.6	601.9	107.7	392.4	2157.9	≥50	≥50	14.5	18.2

Values are the average of 2 experiments.
[1] Peptides quantified: P1 (LQLQPFPQPQLPY); P2 (LQLQPFPQPQLPYPQPQPF); P3 (LQLQPFPQPQLPYPQPQLPYPQPQPF); P4 (LQLQPFPQPQLPYPQPHLPYPQPQPF); P5 (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF); P6 (RPQQPYPQPQPQY); P7 (RPQQPYPQSQPQY); P8 (QQQLIPCRDVVL); and P9 (QQILQQQLIPCRDVVL).

to quantitate total α-gliadin, the values ranged from 2.45 to 823.5. Fig. 2 shows the amount determined for immunogenic epitopes glia-α9 (PFPQPQLPY) and total α-gliadin. Comparatively higher amounts of the epitope glia-α9 were found in cultivars Pilot, Pioneer-2375 and Barlow, released in 1939, 1990 and 2009 respectively. Cultivar Russ (1995), which showed low values in the relative quantification experiment showed the lowest values in the absolute quantification experiment as well, for the glia-α9 immunogenic epitope and the amount of total α-gliadin. Therefore, the results of the two experiments are comparable in terms of identifying the least amount of peptides. The amount of total α-gliadin calculated was lower than the values for immunogenic epitopes because the immunogenic sequences are repeated at a higher frequency, than the sequences used to determine the amount of total α-gliadin. Van den Broeck et al. (2015), also reported similar observations for one of the cultivars that they analyzed, where the amount of total α-gliadin was lower than that of the immunogenic peptides. Additionally, the results show that the total amount of α-gliadin is not associated with the amount of immunogenic epitopes.

The results of the quantification experiments, both relative and absolute, suggest that the amount of immunogenic epitopes and total α-gliadin varied randomly across the hundred years that were analyzed, indicating that there is no relation between release year of cultivar and CD antigenicity. These findings are not in agreement with those of van den Broeck et al. (2015 and 2010), who reported that modern wheat cultivars contain higher amounts of CD epitopes. This discrepancy could be due to wheat genotype variations in the United States and Netherlands. Additionally, Van den Broeck et al. (2015) performed the quantitation study only on three cultivars, whereas the current study investigated 30 cultivars released over the last 100 years. Our results are somewhat in agreement with those of Prandi et al. (2017), since our results suggest that historical wheat is antigenic for those with CD. However, historical wheat was not found to be higher is antigenicity in comparison to modern wheat. Spaenij-Dekking et al. (2005) determined that there is high genetic variation among different wheat species, and that it is possible to identify wheat accessions that are low in the amount of T-cell stimulatory epitope sequences. From our results, cultivar Russ showed the least amount of immunogenic epitopes and total α-gliadin, making it a suitable starting point for breeding wheat cultivars that are safe for CD patients.

4. Conclusion

To summarize, in this study the antigenicity in relation to CD was tested in historical and modern hard red spring wheat cultivars released in North Dakota from 1910 to 2013. Two methods were used in this process. First, RP-HPLC was used to determine how the gliadin profiles changed in relation to release year. And second, mass spectrometric analysis was performed to determine if immunogenic epitopes causing celiac disease are present in the wheat cultivars being investigated, and to quantify these epitopes. Overall, the results indicate that immunogenic epitopes causing CD are detected in historical and modern hard red spring wheat cultivars irrespective of release year, and that the quantity of these epitopes does not show a relationship with cultivar release year, indicating that modern wheat cultivars are not higher in CD antigenicity in comparison to historical wheat cultivars.

5. Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article, and no competing financial interests.

Acknowledgements

The authors thank the members of the Center for Mass Spectrometry and Proteomics, University of Minnesota, United States for their assistance in method development and analysis. The authors also thank Dr. Jae-Bom Ohm for his assistance with statistical analysis.

This work was supported by growers through checkoff funds from the North Dakota Wheat Commission.

References

Bonomi, F., Iametti, S., Mamone, G., & Ferranti, P. (2013). The performing protein: Beyond wheat proteomics? *Cereal Chemistry, 90*(4), 358–366.

Colomba, M. S., & Gregorini, A. (2012). *Are ancient durum wheats less toxic to celiac patients?* Scientific World Journal: A study of alpha-gliadin from Graziella ra and Kamut8.

Gallagher, E., Gormley, T. R., & Arendt, E. K. (2004). Recent advances in the formulation of gluten-free cereal-based products. *Trends in Food Science & Technology, 15*(3–4), 143–152.

Gessendorfer, B., Koehler, P., & Wieser, H. (2009). Preparation and characterization of enzymatically hydrolyzed prolamins from wheat, rye, and barley as references for the immunochemical quantitation of partially hydrolyzed gluten. *Analytical and Bioanalytical Chemistry, 395*(6), 1721–1728.

Gianfrani, C., Maglio, M., Aufiero, V. R., Camarca, A., Vocca, I., Iaquinto, G., ... Mazzarella, G. (2012). Immunogenicity of monococcum wheat in celiac patients. *American Journal of Clinical Nutrition, 96*(6), 1339–1345.

Gregorini, A., Colomba, M., Ellis, H. J., & Ciclitira, P. J. (2009). Immunogenicity characterization of two ancient wheat alpha-gliadin peptides related to coeliac disease. *Nutrients, 1*(2), 276–290.

Lin-Moshier, Y., Sebastian, P. J., Higgins, L., Sampson, N. D., Hewitt, J. E., & Marchant, J. S. (2013). Re-evaluation of the role of calcium homeostasis endoplasmic reticulum protein (CHERP) in cellular calcium signaling. *Journal of Biological Chemistry, 288*(1), 355–367.

Lookhart, G., & Bean, S. (1995). Separation and characterization of wheat-protein fractions by high-performance capillary electrophoresis. *Cereal Chemistry, 72*(6), 527–532.

MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M., Finney, G. L., Frewen, B., ... MacCoss, M. J. (2010). Skyline: An open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics, 26*(7), 966–968.

Piston, F., Gil-Humanes, J., Rodriguez-Quijano, M., & Barro, F. (2011). Down-regulating gamma-gliadins in bread wheat leads to non-specific increases in other gluten proteins and has no major effect on dough gluten strength. *Plos One, 6*(9), 1–10.

Prandi, B., Mantovani, P., Galaverna, G., & Sforza, S. (2014). Genetic and environmental factors affecting pathogenicity of wheat as related to celiac disease. *Journal of Cereal Science, 59*(1), 62–69.

Prandi, B., Tedeschi, T., Folloni, S., Galaverna, G., & Sforza, S. (2017). Peptides from gluten digestion: A comparison between old and modern wheat varieties. *Food Research International, 91*, 92–102.

Rappsilber, J., Ishihama, Y., & Mann, M. (2003). Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Analytical Chemistry, 75*(3), 663–670.

Salplachta, J., Marchetti, M., Chmelik, J., & Allmaier, G. (2005). A new approach in proteomics of wheat gluten: Combining chymotrypsin cleavage and matrix-assisted laser desorption/ionization quadrupole ion trap reflectron tandem mass spectrometry. *Rapid Communications in Mass Spectrometry, 19*(18), 2725–2728.

Schägger, H., & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem, 166*(2), 368–379.

Shevchenko, A., Wilm, M., Vorm, O., & Mann, M. (1996). Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Analytical Chemistry, 68*(5), 850–858.

Sollid, L. M., Qiao, S. W., Anderson, R. P., Gianfrani, C., & Koning, F. (2012). Nomenclature and listing of celiac disease relevant gluten T-cell epitopes restricted by HLA-DQ molecules. *Immunogenetics, 64*(6), 455–460.

Spaenij-Dekking, L., Kooy-Winkelaar, Y., Van Veelen, P., Drijfhout, J. W., Jonker, H., Van Soest, L., ... Koning, F. (2005). Natural variation in toxicity of wheat: Potential for selection of nontoxic varieties for celiac disease patients. *Gastroenterology, 129*(3), 797–806.

Suligoj, T., Gregorini, A., Colomba, M., Ellis, H. J., & Ciclitira, P. J. (2013). Evaluation of the safety of ancient strains of wheat in coeliac disease reveals heterogeneous small intestinal T cell responses suggestive of coeliac toxicity. *Clinical Nutrition, 32*(6), 1043–1049.

van den Broeck, H. C., Cordewener, J. H. G., Nessen, M. A., America, A. H. P., & van der Meer, I. M. (2015). Label free targeted detection and quantification of celiac disease immunogenic epitopes by mass spectrometry. *Journal of Chromatography A, 1391*, 60–71.

van den Broeck, H. C., de Jong, H. C., Salentijn, E. M. J., Dekking, L., Bosch, D., Hamer, R. J., ... Smulders, M. J. M. (2010). Presence of celiac disease epitopes in modern and old hexaploid wheat varieties: Wheat breeding may have contributed to increased prevalence of celiac disease. *Theoretical and Applied Genetics, 121*(8), 1527–1539.

Wang, S. L., Shen, X. X., Ge, P., Li, J., Subburaj, S., Li, X. H., ... Yan, Y. M. (2012). Molecular characterization and dynamic expression patterns of two types of gamma-gliadin genes from aegilops and triticum species. *Theoretical and Applied Genetics, 125*(7), 1371–1384.

Ward, J. H. (1963). Hierarchical grouping to optimize an objective function. *Journal of the American Statistical Association, 58*(301) 236 000.

Wieser, H., & Koehler, P. (2008). The biochemical basis of celiac disease. *Cereal Chemistry, 85*(1), 1–13.