Specific Nongluten Proteins of Wheat Are Novel Target Antigens in Celiac Disease Humoral Response

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ABSTRACT: While the antigenic specificity and pathogenic relevance of immunologic reactivity to gluten in celiac disease have been extensively researched, the immune response to nongluten proteins of wheat has not been characterized. We aimed to investigate the level and molecular specificity of antibody response to wheat nongluten proteins in celiac disease. Serum samples from patients and controls were screened for IgG and IgA antibody reactivity to a nongluten protein extract from the wheat cultivar Triticum aestivum Butte 86. Antibodies were further analyzed for reactivity to specific nongluten proteins by two-dimensional gel electrophoresis and immunoblotting. Immunoreactive molecules were identified by tandem mass spectrometry. Compared with healthy controls, patients exhibited significantly higher levels of antibody reactivity to nongluten proteins. The main immunoreactive nongluten antibody target proteins were identified as serpins, purinins, α-amylase/protease inhibitors, globulins, and farinins. Assessment of reactivity toward purified recombinant proteins further confirmed the presence of antibody response to specific antigens. The results demonstrate that, in addition to the well-recognized immune reaction to gluten, celiac disease is associated with a robust humoral response directed at a specific subset of the nongluten proteins of wheat.

KEYWORDS: Celiac disease, immune response, antibody, serpin, purinin, α-amylase/protease inhibitor, globulin, farinin

INTRODUCTION

Celiac disease is a prevalent autoimmune disorder, with documented presence in populations of North and South America, Europe, north Africa, south and west Asia, and Australia.1,2 The symptoms of the disease are triggered in genetically susceptible individuals by ingestion of wheat and related cereal proteins of rye and barley. The ensuing innate and adaptive immune responses to the ingested proteins are responsible for inflammation, villous atrophy, and crypt hyperplasia in the small intestine, as well as the production of autoantibodies against the transglutaminase 2 (TG2) enzyme.3 In addition to intestinal symptoms, celiac disease may involve extra-intestinal complications.4 Dermatitis herpetiformis is the skin manifestation of celiac disease, affecting about 10–20% of celiac disease patients and is characterized by papulovesicular lesions and presence of granular deposits of IgA in the dermal papillae.5,6 Elimination of the offending cereals from diet is currently the only effective mode of treatment for celiac disease. Willem Karel Dicke was the first to recognize the importance of the removal of wheat and related cereals from diets of celiac disease patients in 1950.7 Shortly after that, the alcohol-soluble subfraction containing the gliadin proteins was determined to contain the main “toxic factor” in the offending grains.8 Gliadin proteins were found to trigger B and T cell-mediated immune responses, which were thought to play a significant role in the inflammatory cascade in celiac disease.9,10 Antibodies against gliadin proteins were demonstrated to be closely associated...
with celiac disease and were widely utilized as serologic markers of the condition prior to the discovery of anti-TG2 autoantibodies. Antibodies against specific deamidated sequences of gliadin were eventually found to be more specifically associated with celiac disease than those against unmodified native gliadin. In addition to the gliadins, glutenin proteins of wheat, initially thought to be harmless, were later reported to trigger antibody and T cell immune responses in celiac disease patients. Together, gliadins and glutenins comprise approximately 70 different proteins, collectively referred to as gluten. They are the major storage proteins of wheat and related cereals, representing about 75% of the total protein content of wheat grain. An important property of the gluten proteins is their poor solubility in water. The nonglutens comprise the remainder of the wheat proteome and include several minor storage proteins, α-amylase/protease inhibitors, and a variety of other enzymes. These proteins are generally much more soluble in water or aqueous salt solutions than gluten proteins and have been historically referred to as albumins/globulins. Several nonglutens, including α-amylase/protease inhibitor, thiol reductase, serine protease inhibitor (serpin), and β-amylase have been identified as potent allergens in IgE-mediated wheat allergy and/or baker’s asthma. However, the nonglutens of wheat and related cereals generally have been considered to be nontoxic and to lack immunogenic potential in the context of celiac disease. A few studies on small numbers of patients have examined immune reactivity to crude albumin/globulin extracts in celiac disease, with divergent results. The earliest of these investigated antibody activities in 24 untreated celiac disease patients (but no healthy results. The earliest of these investigated antibody reactions in small numbers of patients have examined immune reactivity to wheat, initially thought to be harmless, were later reported to trigger antibody and T cell immune responses in celiac disease patients.

## MATERIALS AND METHODS

### Patients and Controls

Serum samples were from 120 individuals, including 50 patients with celiac disease (18 male, 46 white race, mean [SD] age 44.6 [17.5] years), 20 patients with dermatitis herpetiformis (11 male, 20 white race, mean age 43.1 (20.8) years), and 50 unaffected controls (20 male, 46 white race, mean age 37.1 [11.3] years). All cases of celiac disease were biopsy-proven and diagnosed according to previously described criteria. All patients with dermatitis herpetiformis had biopsies demonstrating classic histology, as well as the characteristic immunofluorescence pattern showing clear granular IgA deposits in the dermal papillae. The celiac disease and dermatitis herpetiformis patients were on a gluten-containing diet. Screening questionnaires were used to evaluate the general health of controls. Serum samples were obtained from patients and healthy controls under institutional review board-approved protocols at Columbia University and at University of Utah. This study was approved by the Institutional Review Board of Columbia University Medical Center. All serum samples were maintained at −80 °C for stability.

### Protein Extraction

One hundred milligrams of the U.S. hard red spring wheat Triticum aestivum Butte 86 flour was suspended in 1 mL of 40% ethanol and mixed for 30 min at room temperature. The suspension was centrifuged at 10,000 × g for 15 min. The supernate was removed, chilled at 4 °C for 1 h, combined with 1.9 mL of 1.5 M NaCl, and stored at 4 °C overnight. The precipitate was removed by centrifugation, rinsed with H2O, and dissolved in 0.2 mL of 0.1 M glacial acetic acid. The solution, containing gluten proteins, was lyophilized and stored at −20 °C.

The nonglutens of Butte 86 wheat flour were extracted as previously described. Fifty milligrams of flour was suspended in 200 μL of buffer (50 mM Tris-HCl, 100 mM KCl, 5 mM EDTA, pH 7.8) at 4 °C and incubated for 5 min with intermittent vortex mixing. Samples were centrifuged at 4 °C for 15 min at 14,500 × g. The supernate was collected, and proteins were precipitated by the addition of 4 volumes of cold (−20 °C) acetone. Following incubation overnight at −20 °C, samples were centrifuged at 14,000 × g for 15 min at 4 °C. The pellet was rinsed with cold acetone, air-dried, and stored at −20 °C.

### Measurement of Antibody Levels

All patients and controls were tested for the currently recommended full panel of the most sensitive and specific serologic markers of celiac disease, including IgA antibody to TG2, IgG antibody to deamidated gliadin, and IgA antibody to deamidated gliadin. IgA antibody to recombinant human TG2 was measured by ELISA, according to the manufacturer’s protocol (Euroimmun AG, Luebeck, Germany). IgG and IgA antibody reactivities to deamidated gliadin, as represented by a previously described glutamine-glutamate substituted trimer of a fusion peptide containing the sequences PLQPEQPFP and PEQLPQFEE, were measured by separate ELISAs, according to the manufacturer’s protocols (Euroimmun AG).
Serum IgG and IgA antibodies to the gluten and nongluten protein extracts were measured separately by ELISA as previously described, with some modifications. Prior to the ELISA analyses, the protein profile of each extract was assessed by SDS-PAGE, using the XCell SureLock Mini-Cell electrophoresis system, 4–12% NuPAGE Bis-Tris precast gels, and 2-N-morpholino)ethanesulfonic acid (MES) buffer (Life Technologies, Carlsbad, Calif.). A 2 mg/mL stock solution of the gluten extract in 70% ethanol or the nongluten protein extract in PBS was prepared. Wells of 96-well Maxisorp round-bottom polystyrene plates (Nunc, Roskilde, Denmark) were coated with 50 μL/well of a 0.01 mg/mL solution of protein extract in 0.1 M carbonate buffer (pH 9.6) or left uncoated to serve as controls. After incubation at 37 °C for 1 h, all wells were washed and blocked by incubation with 1% bovine serum albumin (BSA) in PBS containing 0.05% Tween-20 (PBST) for 1.5 h at room temperature. Serum samples were diluted at 1:200 for IgA and at 1:800 for IgG measurement, added at 50 μL/well in duplicate, and incubated for 1 h. Each plate contained a positive control sample from a patient with biopsy-proven celiac disease and elevated IgG and IgA antibodies to each protein extract. After washing, the wells were incubated with HRP-conjugated antihuman IgG (GE Healthcare, Piscataway, N.J.) or IgA (MP Biomedicals, Santa Ana, Calif.) secondary antibodies for 50 min. The plates were washed and 50 μL of developing solution, containing 27 mM citric acid, 50 mM Na2HPO4, 5.5 mM o-phenylenediamine, and 0.01% H2O2 (pH 5), was added to each well. After incubating the plates at room temperature for 20 min, absorbance was measured at 450 nm. All serum samples were tested in duplicate. Absorbance values were corrected for nonspecific binding by subtraction of the mean absorbance of the associated uncoated wells. The corrected values were first normalized according to the mean value of the positive control duplicate on each plate. The mean antibody level for the unrelated healthy control cohort was then set as 1.0 AU, and all other results were normalized accordingly.

Two-Dimensional Gel Electrophoresis

The KCl-soluble proteins were separated by two-dimensional electrophoresis as previously described. The dried protein was solubilized at 1.2 mg/mL in a solution containing 9 M urea, 4% Nonidet P-40, 1% dithiothreitol, and 2% Servalyt 3–10 IsoDalt (Crescent Chemical Co., Islandia, N.Y.). The first dimension capillary tube gels contained 9.2 M urea, 4% (total mono- mer) acrylamide/Bis, 2% Nonidet P-40, 2% Servalyt 3–10 Iso-Dalt, 0.015% ammonium persulfate, and 0.125% TEMED. Isoelectric focusing was performed using a Mini Protean II Iso-Dalt, 0.015% ammonium persulfate, and 0.125% TEMED. The KCl-soluble proteins were separated by two-dimensional electrophoresis as previously described. The dried protein was solubilized at 1.2 mg/mL in a solution containing 9 M urea, 4% Nonidet P-40, 1% dithiothreitol, and 2% Servalyt 3–10 IsoDalt (Crescent Chemical Co., Islandia, N.Y.). The first dimension capillary tube gels contained 9.2 M urea, 4% (total monomer) acrylamide/Bis, 2% Nonidet P-40, 2% Servalyt 3–10 Iso-Dalt, 0.015% ammonium persulfate, and 0.125% TEMED. Isoelectric focusing was performed using a Mini Protean II Iso-Dalt, 0.015% ammonium persulfate, and 0.125% TEMED. The KCl-soluble proteins were separated by two-dimensional electrophoresis as previously described.

Immunoblotting

Protein transfer onto nitrocellulose membranes was carried out with the iBlot Dry Blotting System (Life Technologies). The membranes were incubated for 1 h in a blocking solution made of 5% milk and 0.5% BSA in a solution of Tris-buffered saline containing 0.05% Tween-20 (TBST). Incubation with patient and control serum specimens (1:2000 for IgA and 1:4000 for IgG determination in dilution buffer containing 10% blocking solution and 10% fetal bovine serum in TBST) was done for 1 h. Serum samples from celiac disease (n = 14) and dermatitis herpetiformis (n = 6) patients with elevated IgA and/or IgG antibody reactivity to nongluten proteins, in addition to 5 healthy controls, were included. HRP-conjugated antihuman IgA and IgG were used as secondary antibodies. Detection of bound antibodies was by the ECL system (Millipore, Billerica, Mass.) and autoradiography film (Crystal- gen, Commack, N.Y.). Following immunodetection, bound antibodies were removed from the nitrocellulose membranes with Restore Western blot stripping buffer (Thermo Scientific, Rockford, Ill.), and the membrane proteins were visualized using colloidal gold stain (Bio-Rad). Each immunoblot was aligned to its corresponding colloidal gold-stained membrane using the SameSpots software (version 4.5) (TotalLab Ltd., Newcastle upon Tyne, United Kingdom).

Identification of Target Proteins

Proteins in the two-dimensional electrophoresis spots that were the main targets of the antibody response were identified initially by comparison to a previously generated proteomic map of Butte 86 flour. Identities of individual spots were then confirmed by MS/MS. Spots were excised from gels and placed in wells of a 96-well reaction plate, leaving a blank well between each sample. Proteins in each sample-well were reduced, alkylated, and then digested with trypsin using a DigestPro instrument (Intavis, Koeln, Germany) according to the manufacturer’s instructions. The resulting tryptic peptides were eluted into a collection tray that was then placed into the autosampler compartment of an EASY-nLC II (Thermo Scientific, Waltham, Mass.) that was interfaced by a nanoelectrospray source to an Orbitrap Elite mass spectrometer (Thermo Scientific). Four microliter fractions were loaded by the autosampler onto an IntegraFrit trap column (100 μm × 200 mm, with 5 μm, 120 Å, ReproSil-Pur C18 AQ packing) (New Objective, Woburn, Mass.), and the membrane proteins were visualized using colloidal gold stain (Bio-Rad). Each immunoblot was aligned to its corresponding colloidal gold-stained membrane using the SameSpots software (version 4.5) (TotalLab Ltd., Newcastle upon Tyne, United Kingdom).

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Protein transfer onto nitrocellulose membranes was carried out with the iBlot Dry Blotting System (Life Technologies). The membranes were incubated for 1 h in a blocking solution made of 5% milk and 0.5% BSA in a solution of Tris-buffered saline containing 0.05% Tween-20 (TBST). Incubation with patient and control serum specimens (1:2000 for IgA and 1:4000 for IgG determination in dilution buffer containing 10% blocking solution and 10% fetal bovine serum in TBST) was done for 1 h. Serum samples from celiac disease (n = 14) and dermatitis herpetiformis (n = 6) patients with elevated IgA and/or IgG antibody reactivity to nongluten proteins, in addition to 5 healthy controls, were included. HRP-conjugated antihuman IgA and IgG were used as secondary antibodies. Detection of bound antibodies was by the ECL system (Millipore, Billerica, Mass.) and autoradiography film (Crystal-gen, Commack, N.Y.). Following immunodetection, bound antibodies were removed from the nitrocellulose membranes with Restore Western blot stripping buffer (Thermo Scientific, Rockford, Ill.), and the membrane proteins were visualized using colloidal gold stain (Bio-Rad). Each immunoblot was aligned to its corresponding colloidal gold-stained membrane using the SameSpots software (version 4.5) (TotalLab Ltd., Newcastle upon Tyne, United Kingdom).

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match instrument-generated spectra to FASTA sequences in the “SuperWheat” database. For the first and second pass search, the parent mass tolerance was set to 15 ppm and the fragment error to 0.4 Da. Charge state screening allowed +1, +2, and +3 charge states to be selected, and one missed cleavage was allowed. Analysis, validation, and display of the data were carried out using Scaffold version 4.3.2 [http://www.proteomessoftware.com]. The output results files from the two different search engines were combined in individual folders and analyzed as separate MudPIT experiments. A second database of FASTA files was generated from the first pass search by exporting from Scaffold all proteins that contained one peptide hit and had a protein probability of 20% and peptide probability of 0%. A reverse-concatenated database was created from these sequences. The resulting subset database, containing 14,776 sequences, was used for the second pass search. Criteria for protein acceptance in the second pass search was set in the Scaffold validation software package to a protein probability of 99% and a requirement for four matching peptides having a parent peptide mass accuracy of 2 ppm and a calculated 95% probability. The list of proteins identified in each spot is shown in Supplemental File 2. The protein with the greatest number of exclusive unique spectra was deemed to be the most abundant in each spot. Peptide data for the predominant protein in each spot were extracted from Scaffold. Sequence coverages determined by Scaffold were adjusted for the presence of the signal peptides as predicted by SignalP 4.1 [http://www.cbs.dtu.dk/services/SignalP/]. The data files associated with this study were archived at UCSD Center for Computational Mass Spectrometry in the MassIVE data set project. The raw mass spectrum files, the sequence database files, and the Scaffold results report can be downloaded from ftp://MSV00078887:a@massive.ucsd.edu/. In order to visualize Scaffold report results, a free viewer can be downloaded from http://www.proteomessoftware.com/products/free-viewer. To visualize the instrument raw files, a viewer can be downloaded from the ProteoWizard project at http://proteowizard.sourceforge.net/downloads.shtml. A suitable text editor for viewing the database files is available at http://www.vim.org/download.php.

Expression of Recombinant Proteins

The cDNAs encoding the identified serpin protein in spot 1a (GI: 224589270) and the purinin protein in spot 2a (BU_purinin#3) (Table 2) were synthesized (after codon optimization for an E. coli expression system) based on amino acid sequences shown in Supplemental File 4. For the purinin, a potential signal peptide cleavage site was detected at position 19 and amino acids 1−19 were therefore excluded. The synthesized genes were inserted into the restriction sites NdeI and HindIII of the tag-free vector E3 to create the E. coli expression vectors E3-224589270 and E3-BU_purinin#3. Linearized vectors were transformed into E. coli, and protein expression was induced by adding 100 mM isopropyl 1-thiogalactopyranoside to the culture for 4 h at 37 °C. Cells were lysed in lysis buffer (50 mM Tris, 1 mM PMSF, pH 8.0), and inclusion bodies, containing insoluble proteins, were collected for purification. After washing (50 mM Tris, 1% Triton X-100, 300 mM NaCl, 2 mM DTT, 1 mM EDTA, pH 8.0), pellets were dissolved in 8 M urea. The purified proteins were suspended in refolding buffer (50 mM Tris, 10% glycercor, 150 mM NaCl, pH 8.0). The molecular weight and relative purity of the proteins were assessed by SDS-PAGE, following the above protocol. The identity of each protein was confirmed by mass spectrometry-assisted peptide mass mapping, as previously explained.30

Antibody Reactivity to Recombinant Proteins

Antibody reactivity to the generated recombinant serpin and purinin proteins was assessed by immunoblotting. Recombinant proteins (0.2 μg) were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The immunoblotting protocol was as described above. Detection of bound HRP-conjugated secondary antibodies was by ECL and the FluoroChem M imaging system (Protein Simple, Santa Clara, Calif.).

Data Analysis

Differences between groups were analyzed by parametric or nonparametric one-way analysis of variance (ANOVA) with posthoc testing for multiple comparisons. Differences with p values of <0.05 were considered to be statistically significant. Statistical analyses were performed with Prism 6 (GraphPad, San Diego, Calif.).

RESULTS

Measurement of Antibody Levels

IgA antibody to TG2 and IgG/IgA antibodies to deamidated gliadin, which are considered to be highly specific and sensitive for celiac disease, were measured in all patients and controls. Celiac disease and dermatitis herpetiformis groups displayed significantly higher mean levels of IgA anti-TG2 antibody (Figure 1A), IgA antideamidated gliadin antibody (Figure 1B), and IgG antideamidated gliadin antibody (Figure 1B) than those of healthy controls (p < 0.0001 for all comparisons).

![Figure 1](https://example.com/fig1.png)

Figure 1. Mean levels of antibody to (A) human TG2 (IgA) and (B) deamidated gliadin fusion peptide (IgA and IgG) in patients with celiac disease and dermatitis herpetiformis in comparison with unaffected controls, as determined by ELISA. Error bars represent the standard error of the mean.

The gel electrophoresis profiles of the gluten and nongluten protein extracts used for the ELISA analyses are shown in Figure 2A. Levels of IgA and IgG class antibodies to the extracted gluten and nongluten protein fractions were measured in all patients and controls. Compared with healthy controls, the two patient groups displayed significantly higher mean levels of IgA (p < 0.0001 for celiac disease; p < 0.001 for dermatitis herpetiformis) and IgG (p < 0.0001 for both) antibody to gluten proteins (Figure 2B). In addition, compared with healthy controls, the celiac disease and dermatitis herpetiformis patient cohorts exhibited significantly increased...
serum IgA ($p < 0.0001$ for both) and IgG ($p < 0.0001$ for both) antibody reactivity to the proteins of the nongluten extract (Figure 2C).

**Immunoblotting and Identification of Target Proteins**

In order to identify the molecular targets of the detected increased antibody response to nongluten proteins in celiac disease and dermatitis herpetiformis patients, a randomly selected subset of antibody-positive sera was further analyzed. Antibody reactivity was characterized by immunoblotting following two-dimensional separation of the nongluten proteins of the wheat cultivar Butte 86. The two-dimensional electrophoresis pattern of the nongluten proteins following Coomassie staining is shown in Figure 3A. The immunoblotting analyses demonstrated antibody reactivity to specific nongluten proteins of wheat in 19 of 20 analyzed patient sera. These included 10 of 11 celiac disease and 2 of 3 dermatitis herpetiformis samples tested for IgA reactivity, and 9 of 9 celiac disease and 6 of 6 dermatitis herpetiformis samples tested for IgG reactivity. None of the control sera exhibited antibody binding to any proteins at the dilutions and exposures tested. Immunoblot images from representative patients and controls are shown in Figure 3B–G.

Proteins within five regions of the two-dimensional gels reacted with patient sera (Figure 3). Comparison of the positions of the reactive proteins with a proteomic map of Butte 86 flour suggested that the reactive proteins belonged to the following distinct protein groups: serpins, purinins, $\alpha$-amylase/protease inhibitors, globulins, and farinins (Figure 3 and Table 1). Identities of proteins in individual spots that reacted with sera were verified by MS/MS using an Orbitrap Elite mass spectrometer and subsequent database search. The generated data files are archived as described in Materials and Methods. The predominant protein in each target spot is reported in Table 2. Peptide data for the predominant protein in each spot are displayed in Supplemental File 3, along with the sequence coverage of each protein. Supplemental File 4 contains the amino acid sequences of the identified target proteins from Table 2. Because of the sensitivity of the instrument, the analysis indicated that most spots contained multiple proteins (Supplemental File 2). However, the majority of the spectra obtained from each spot corresponded to a specific protein that was deemed the predominant protein in the spot (Table 2 and Supplemental Files 2 and 3). The MS/MS data confirmed the presence of the five protein types that had been initially identified through comparison of spot positions with the proteomic map of Butte 86 flour. For spots 2c and 2d, the MS/MS data indicated that the spots contained purinins proteins (Butte purinin#1 and Butte purinin#2) (Supplemental Files 2 and 3 and Table 2), even though nonpurinin type proteins in those spots had the highest exclusive unique spectrum count.

Description of results according to patient group is presented in Tables 1 and 2. Patients were most frequently reactive to serpins (75% of tested celiac disease and dermatitis herpetiformis patient samples), followed by purinins (65%), $\alpha$-amylase/protease inhibitors (60%), globulins (40%), and farinins (35%). The most frequently reactive individual protein spot was identified as a serpin (spot 1a in Figure 3A; GI: 224589270) (Table 2). Fifteen of the 20 patients (75%) and none of the controls exhibited antibody reactivity to this protein spot. Within the purinin group, the most reactive individual protein spot (2a) was identified as BU_purinin#3; 10 of 20 patients (50%) displayed antibody reactivity to it. Frequencies of reactivity to the identified proteins did not appear to be substantially different between the celiac disease and dermatitis herpetiformis groups.

**Antibody Reactivity to Recombinant Proteins**

In order to further confirm the presence of antibody reactivity to selected nongluten antigens identified in this study, we generated proteins by recombinant expression based on the available amino acid sequences for the serpin in spot 1a and the purinin in spot 2a. Immunoblotting with the recombinant proteins confirmed the presence of IgG and/or IgA antibody reactivity to the specific serpin and purinin proteins in patients with celiac disease or dermatitis herpetiformis who had been found to be positive for antibodies to spot 1a and/or spot 2a (Figure 4). Healthy controls did not display any reactivity at the serum dilutions and image acquisition exposures used.

**DISCUSSION**

Heightened adaptive immune response to ingested gluten proteins of wheat and related cereals is a hallmark of celiac disease. A few earlier studies, carried out on small numbers of celiac disease patients, had attempted to assess immune reactivity to nongluten proteins of wheat. 22–26 However, the results were inconsistent, and the purity of the antigenic...
mixture in the crude nongluten protein extracts used for detecting immune reactivity was later questioned. Here, we have demonstrated the presence of a robust humoral response to specific nongluten proteins of wheat in patients with celiac disease and dermatitis herpetiformis. The detailed analysis of antibody reactivity through two-dimensional immunoblotting and MS/MS identification of target proteins provides unequivocal confirmation that the main targets of the antibody response are, in fact, specific proteins that have not been categorized as gluten or previously recognized as triggers of humoral response in celiac disease. A serpin protein was the most reactive nongluten antigen identified. Wheat serpin proteins belong to the large family of serine protease inhibitors that are present in many organisms, most of them acting as suicide substrate inhibitors of chymotrypsin-like proteases. Other nongluten target antigens of wheat included purinins, α-amylase/protease inhibitors, globulins, and farinins.

The mechanism responsible for generating antibodies against proteins that are generally thought of as nontoxic in the context of celiac disease is not clear. The elevated antibody reactivity to nongluten proteins may be driven by the enhanced inflammatory environment in the gut, brought on by the villous damage and ensuing epithelial barrier dysfunction that are triggered by gluten in celiac disease. However, it should be noted that we did not detect antibodies against a broad array of nongluten proteins. For example, no antibody reactivity to triticin proteins, which are present at similar or higher concentration in wheat flour as serpins, globulins, farinins, and purinins, was found.

Another possibility is that the peripheral antibody response to gluten proteins in celiac disease may cross-react with specific nongluten antigens that contain similar epitopes, thus contributing to the detected levels of antibodies against nongluten proteins. A homology analysis indicates that the newly named purinun proteins are close in sequence to γ-gliadins.
In addition, there are short sequences in the identified \(\alpha\)-amylase/protease inhibitors and another newly characterized group of proteins, the farinins,\(^{34}\) which are similar to those in certain \(\gamma\)-gliadin and low molecular weight glutenin proteins. In addition, the reactive centers of some of the identified serpin antigens resemble the glutamine-rich repeats in gluten proteins.\(^{55}\) Whether these particular shared sequences are actually immunogenic and capable of contributing to the presence of cross-reactive antibodies, however, is not obvious at this point and needs further investigation.

\[\text{dx.doi.org/10.1021/pr500809b J. Proteome Res. 2015, 14, 503–511}\]
The observed increase in IgA and IgG antibody responses to specific nongluten proteins brings up the obvious question of whether the identified immune response might be relevant to the pathogenic mechanism of celiac disease. Similar to gluten proteins, serpins and α-amylase/protease inhibitors are resistant to proteolytic digestion and are therefore likely to be present in the form of long stretches of incompletely digested, and potentially immunogenic, sequences in the small intestine. The observed IgA, along with IgG, reactivity to the identified nongluten proteins points to a mucosal origin for the immune-triggering event. However, it remains to be seen whether the mucosal B cell response is accompanied by a corresponding intestinal CD4+ T cell reactivity to the nongluten proteins. Presence of lamina propria CD4+ T cells with specificity for sequences of the identified nongluten proteins may contribute to the celiac disease-associated pathways in the gut, for example, by providing additional help to the autoreactive CD8+ intraepithelial cytotoxic T cells that drive epithelial cell damage. It is also possible that the detected antibodies to nongluten proteins would themselves contribute to the mucosal lesion. Similar to the effect shown for antigluten antibodies, the antibodies against the nongluten proteins may be involved in inducing local complement activation and mucosal damage.36 They may also contribute to recruitment of various leukocytes that lead to antibody-dependent cell-mediated cytotoxicity, a process that has been previously demonstrated for antibodies to gluten.37 However, the observed immune response to nongluten proteins, as was mentioned earlier, may be the result and a bystander marker of intestinal barrier damage and inflammation, without playing a role in the pathogenic mechanism of celiac disease.

In conclusion, the results of this study clearly demonstrate that the humoral response to wheat in celiac disease is not limited to gluten antigens but is also directed at specific nongluten proteins. While direct conclusions cannot be drawn about the pathogenic effects of the identified nongluten proteins, these findings should prompt further research into their potential role in contributing to the inflammatory processes that result in mucosal damage in patients with celiac disease. The possibility of such a role for these proteins is worthy of attention, especially as therapies other than gluten exclusion from the diet are under development.38 For example, proteolytic enzymes with specificity for the toxic fragments of gluten,39 may be insufficiently active against other immunogenic proteins. In addition, further investigation of the utility of the identified antibodies as potential biomarkers in celiac disease or other gluten-related disorders may be warranted.

**ASSOCIATED CONTENT**

Supporting Information

Supplemental file 1. Translated sequences of 137 ESTs, contigs, or PCR products from Butte 86 that were included in the “SuperWheat” database. Supplemental file 2. Data extracted from Scaffold showing the proteins identified in each spot. Supplemental file 3. Peptide data and sequence coverage for the predominant protein in each spot. Supplemental file 4. Amino acid sequences of the identified target proteins from Table 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We would like to thank Dr. Carolina Arguelles-Grande and Ms. Maria Minaya of Columbia University for their involvement in specimen collection and organization. We are grateful to the research participants involved. Mention of trade names or commercial products in this report is solely for the purpose of providing specific information and does not imply endorsement by the USDA. The USDA is an equal opportunity provider and employer. Funding was provided by National Center for Advancing Translational Sciences, NIH, through Grant Number U11 TR000040. Study sponsor had no role in study design or in collection, analysis, and interpretation of data.

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