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ORIGINAL ARTICLE

# A combined biochemical, biophysical and immunological approach towards the identification of celiac disease-specific wheat antigens

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**Abstract** Celiac disease (CD) is an inflammatory affliction of the small bowel caused by an immunological hypersensitivity to ingested wheat antigens affecting almost 1 % of the population. The gliadin fraction of wheat has been shown to contain the pathogenic antigens which react with antibodies and T cells. However, there is only limited knowledge regarding the precise nature of the wheat antigens recognized by IgA antibodies from CD patients and diagnostic tests based on the gliadin fraction have been demonstrated to give frequently false positive results. The aim of this study was the characterization of wheat antigens specifically recognized by IgA antibodies of CD patients. We developed a combined biochemical, biophysical, and immunological approach for the identification of celiac disease-specific wheat antigens. It is based on sub-fractionation of the wheat gliadin fraction using two ion exchange chromatography steps, the localization of CD-specific antigens by immunoblotting with IgA antibodies from CD patients, subsequent digestion followed by electro spray ionization–liquid chromatography/mass spectrometry (LC–ESI–MS/MS) and N-terminal sequencing by Edman degradation. Through the sub-fractionation procedure it was possible to separate CD-specific IgA-reactive wheat antigens from other wheat antigens which were also recognized by IgA antibodies of individuals without CD or by CD patients on gluten-free diet. Analysis by LC–ESI–MS/MS and N-terminal sequencing of the sub-fractions and the proteins specifically recognized by CD patients identified certain γ-gliadins with molecular mass of 37,000 and 45,000 as CD-specific wheat antigens. The CD-specific γ-gliadins with the molecular mass of 37,000 and 45,000 should be useful to study pathomechanisms of the disease and to improve the specificity of diagnostic tests for CD.

**Keywords** Wheat · Celiac disease · Antigens · Ion exchange chromatography · Mass spectrometry

**Abbreviations**

| | |
|---|---|
| CD | Celiac disease |
| LC–ESI–MS/MS | Liquid chromatography–Electro spray Ionization/Mass spectrometry |
| GFD | Gluten-free diet |
| AGA | Anti-gliadin antibodies |
| tTG | Tissue transglutaminase |
| DGP | Deamidated gliadin peptide |
| IEC | Ion exchange chromatography |
| SP | Sulphopropyl resin |
| DEAE | Diethylaminoethyl resin |
| FT | Flow through |
| Elu | Elution |
| PVDF | Polyvinylidene fluoride |

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## Introduction

Celiac disease (CD) is a chronic inflammatory small intestinal disorder which is elicited and triggered by a hypersensitive immune response to dietary wheat gluten, especially to the gliadin fraction (Bischoff et al. 2000; Dieterich et al. 2003). Gluten from related cereals, such as rye and barley are also toxic to CD patients. CD affects more than 1 % of the population and is one of the most important wheat-related diseases (Sapone et al. 2012). The harmful effects of dietary wheat gluten on children's health was first recognized by the Dutch paediatrician Dr. Willem-Karel Dicke who also introduced gluten-free diet (GFD) as an effective treatment for CD (Van De Kamer et al. 1953). CD patients display strong genetic affiliation to certain HLA DQ genes (Sollid et al. 1989) and are frequently associated with other autoimmune disorders (Maki et al. 1984).

Wheat gluten which causes CD is a complex aggregate of protein subunits called the gliadins and glutenins, they are rich in Proline (P) and Glutamine (Q) residues. The alcohol-soluble gliadins of wheat are thought to contain the major immunogenic component of gluten. Gliadins can be classified based on their genetic and chemical characteristics into three subtypes, α/β-gliadins, γ-gliadins, and ω-gliadins (Woychik et al. 1961; Wieser 1996). The α/β- and γ-gliadins have similar molecular masses of 28,000–35,000 and differ in the contents of certain amino acids, such as tyrosine. The N-terminal domains of α/β-gliadins and γ-gliadins contain Proline (P) and Glutamine (Q) rich sequences, QPQPFPQQPYP repeats are found in the former and QPQQPFP repeats are found in the latter. Many modifications and variations in the sequence of gliadins occur due to additions/substitutions of few amino acids, leading to great variety of these proteins. The variety of sequences may be also due to the fact that gluten proteins are storage proteins without a biologically active structure, and therefore may not be under evolutionary pressure to prevent variations in their sequences. The C-terminal domains of α/β and γ-gliadins are homologous and contain non-repetitive sequences. PQQPFPQQ sequences represent the repetitive domain in ω-gliadins which have higher molecular masses between 40,000 and 60,000 (Wieser 2007).

One of the major hallmarks of CD is the presence of $CD4^+$ T cells infiltrating the small intestinal mucosa and it has been possible to isolate HLA DQ2/DQ8 restricted, IFN-γ-secreting, gliadin-reactive T cells from CD mucosa (Lundin et al. 1993; Przemioslo et al. 1995; Nilsen et al. 1995). The increased IFN-γ observed in CD mucosa is thought to be a key factor in mucosal damage, villous atrophy, crypt hyperplasia and activation of B cells to produce anti-gliadin antibodies mainly of the IgA isotype, as well as antibodies against proteins of the endomysium and tissue transglutaminase (tTG) which disappear on GFD (Sollid 2002; Skerritt et al. 1987; Natter et al. 2001; Constantin et al. 2005). Interestingly, several studies showed that deamidation of Pro/Gln-rich gliadin-derived peptides by the autoantigen tTG boosts the activation of HLA DQ2-restricted $CD4^+$ T cells (Molberg et al. 1998; van de Wal et al. 1998a, b; Vader et al. 2002; Arentz-Hansen et al. 2000, 2002; Tollefsen et al. 2006; Camarca et al. 2009). It has also been shown that innate immune response to gliadin peptides from Pro/Gln poor regions of gliadin can lead to epithelial cell death (Maiuri et al. 2003; Meresse et al. 2004; Hue et al. 2004). Gliadin-derived peptides are thus capable of inducing adverse immune reactions in genetically susceptible individuals. Hence, the biochemical nature of wheat antigens that activate and sustain the immune response in CD has been a subject of extensive research.

Several studies have identified peptides derived from gluten which are involved in CD (Sollid et al. 2012), but the individual wheat antigens have not been characterized in detail at the biochemical level. Biochemical approaches to enrich different gliadin proteins from whole gliadin extracts is hampered by the fact that there are more than 150 genes coding for gliadins (Anderson and Greene 1997; Qi et al. 2009), which result in large number of expressed gliadins (Lafiandra et al. 1984) and high variability in the composition and length of these proteins. One elegant study describes a reversed-phase high-pressure liquid chromatography-based separation of different gliadins, but a detailed analysis of these protein fractions regarding IgA reactivity with sera from CD patients was not performed (Wieser et al. 1998). Thus, there is only limited knowledge regarding the precise nature of wheat antigens specifically recognized by CD patients. Therefore, we used an immuno-proteomic approach for identifying CD-specific antigens based on biochemical fractionation and the use of patients IgA antibodies.

For this purpose, we developed a method for sub-fractionation of gliadins extracted from wheat seeds, to enrich and identify antigens which are specifically recognized by IgA from CD patients. A combination of biochemical fractionation techniques i.e., ion exchange chromatography (IEC), IgA immunoblotting, Nano HPLC, LC–ESI–MS/MS, and N-terminal protein sequencing was then used to identify CD-specific antigens.

## Materials and methods

### Human sera

Sera from patients suffering from CD ($n = 35$) who have been diagnosed according to ESPGHAN guidelines

(Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition 1990) and positive serology to tissue transglutaminase (tTG) and deamidated gliadin peptide (DGP) when tested with QUANTA Lite® ELISA diagnostic kits (Inova Diagnostics, San Diego, CA) have been used for IgA testing. Eight of these patients had been on GFD for at least 4 months when serum samples were collected, whereas the remaining patients were freshly diagnosed and at that time of serum collection on a gluten-containing diet. Furthermore, sera from healthy individuals ($n = 10$) and two individuals with false positive AGA tests who regularly ate wheat products and did not suffer from CD were tested. Sera from the latter individuals were tested negative with tTG and DGP QUANTA Lite® ELISA diagnostic kits (Inova Diagnostics, San Diego, CA). Sera had been taken in the course of routine diagnostic procedures. The serum samples used for the immunological assays were remaining serum samples which were tested in an anonymized and retrospective manner with permission from the Ethical Committee of the Medical University of Vienna.

### Preparation of gliadin extract

Gliadin fraction was prepared from wheat seeds (*Triticum aestivum* L. Grandios) obtained from the Austrian Agency for Health and Food Safety (AGES) following the modified Osborne procedure (Weiss et al. 1993). For this purpose 10 g of wheat seeds were ground in a small pulveriser and resuspended in 100-ml buffer containing 50-mmol/L Tris pH 8.0, 500-mmol/L NaCl. After incubation for 1 h under continuous mixing at 4 °C, the aqueous-soluble wheat proteins were removed by centrifugation at 21,000×*g* for 30 min to obtain an albumin/globulin fraction. The pellet was washed by repeating the previous steps twice with the same buffer and once with deionised water. Finally, the pellet was resuspended in 25 ml of 70 % ethanol for 2 h and extracted under continuous mixing. The alcohol-soluble gliadin fraction was obtained by centrifugation at 21,000×*g* and termed gliadin fraction (e).

### Sub-fractionation of the gliadin fraction

For IEC, the gliadin fraction (e) was dialysed against buffer A containing 50 mmol/L Tris buffer pH 4.5 and 4 mol/L urea. A sulfopropyl (SP) Sepharose, Hi Trap™ column (GE Healthcare, Uppsala, Sweden) connected to the FPLC (ÄKTA prime, Amersham Biosciences, Watford, USA) was pre-equilibrated with buffer A and 50 ml of gliadin fraction (e) concentration 0.5 mg/ml was injected at a flow rate of 1 mL/min. The flow-through (FT) fraction as monitored by a single path ultraviolet monitor at 280 nm was collected and labeled as sub-fraction FT SP. The column was then washed with 15 ml of buffer A and column-bound proteins were eluted using 25-ml buffer A containing a linear salt gradient of 0–500 mmol/L NaCl, flow rate 1 mL/min. This elution sub-fraction was designated as Elu SP. Twenty five ml of the FT SP sub-fraction was further dialysed against buffer B containing 50 mmol/L Tris pH 10.0 and 4 mol/L urea and injected onto a diethylaminoethyl (DEAE) Sepharose, Hi Trap™ column (GE Healthcare) equilibrated with buffer B at a flow rate of 1 mL/min. The FT fraction was collected and designated sub-fraction FT DEAE. The column was then washed with 15 ml of buffer B and column-bound proteins were eluted with 25 ml buffer B containing a linear salt gradient of 0–500 mmol/L NaCl at a flow rate of 1 mL/min. The elution sub-fraction was labeled as sub-fraction Elu DEAE. The protein contents in each of the fractions and sub-fractions were quantified using a BCA protein Assay kit (Pierce, Rockford, IL) according to manufacturer's instructions.

### Specific rabbit antibodies

Antibodies specific for 1-Cys-peroxiredoxin, a wheat allergen belonging to the water-soluble albumin/globulin fraction of wheat (Pahr et al. 2012) were obtained by immunization of a New Zealand white rabbit with the purified recombinant allergen (200 µg per injection) using once Freund's complete adjuvant and twice incompletes Freund's adjuvant (Charles River, Kisslegg, Germany). Pre-immune serum was obtained from the rabbit before immunization.

### SDS-PAGE and Immunoblotting with patient's serum IgA and detection of 1-Cys-peroxiredoxin with specific rabbit antibodies

Ten µg/well (Figs. 1b, 2a, b, 3) or 20 µg/cm (Fig. 2c) of proteins prepared in SDS sample buffer containing 10 % v/v glycerin, 60 mmol/L Tris/HCl pH 6.8, 2 % w/v SDS, 0.01 % w/v bromophenol blue and boiled for 5 min were separated on a self-cast analytical gel (size 86 × 77 × 1 mm) comprised of stacking gel (5 % T, 2.67 % C, 125 mmol/L Tris/HCl, 0.1 % w/v SDS, pH 6.8) and resolving gel (10 % T, 2.67 % C, 375 mmol/L Tris/HCl, 0.1 % w/v SDS, pH 8.8) mounted on a vertical SDS gel apparatus (Biometra GmbH, Goettingen, Germany). After electrophoresis, proteins were either stained using Coomassie brilliant blue R-250 or subsequently transferred onto a 0.2 µm Whatmann Protran™ nitrocellulose

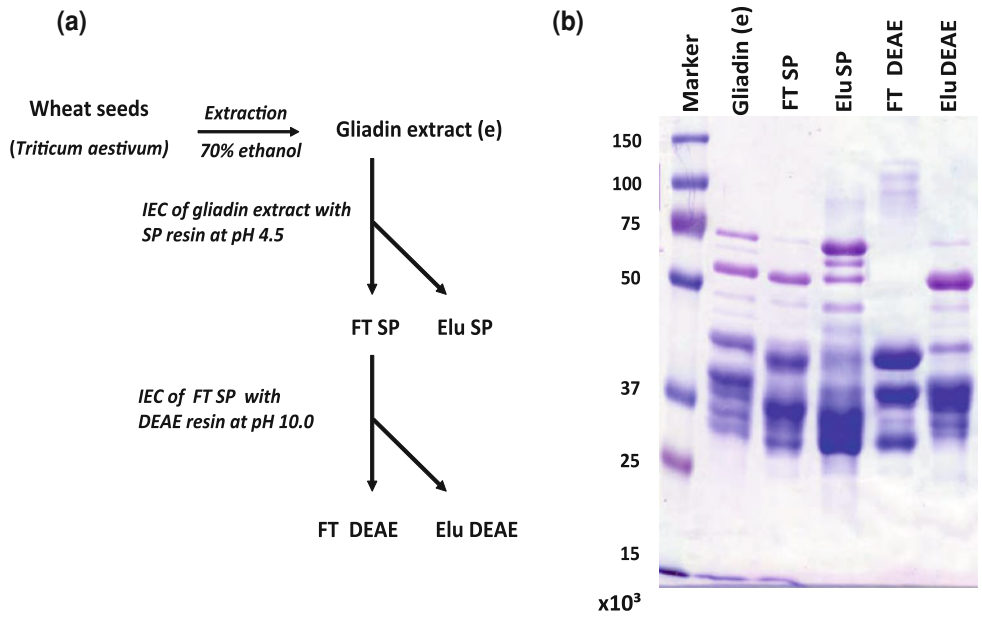



**Fig. 1** Gliadin sub-fractionation using ion exchange chromatography (IEC). Schematic representation **a**. The alcohol-extracted gliadin fraction was first sub-fractionated using a cation exchange SP column sulfopropyl column into a flow-through (FT SP) and elution sub-fraction (Elu SP). In the second step, FT SP was further separated using an anion exchange DEAE column into another flow-through (FT DEAE) and elution sub-fraction (Elu DEAE). Coomassie-stained SDS-PAGE of gliadin extract and gliadin sub-fractions **b**. Lanes contain the fractions and sub-fractions as labeled in **a** and molecular mass $\times 10^3$ of the protein standard is shown on the *left*

membrane by tank blotting method with buffer containing 25 mmol/L Tris, 192 mmol/L Glycine, and 20 % v/v methanol and applying 3.7 mA/cm$^2$ current for 55 min at 4 °C (Laemmli 1970; Towbin et al. 1979). The membranes were blocked in buffer C (50 mmol/L sodium phosphate (pH 7.5), 0.5 % w/v BSA, 0.5 % v/v Tween-20 and 0.05 % w/v sodium azide) twice for 10 min and once for 30 min or 5 mm strips of electroblotted nitrocellulose membranes were cut before blocking. After blocking, the membranes were then incubated overnight at 4 °C with either human sera (from CD patients or control individuals) diluted 1:100 or with rabbit antibodies diluted 1:1000. After washing the membranes with buffer C, bound human IgA antibodies were detected by incubating the blots for 4 h at 4 °C with a purified mouse monoclonal anti-human $IgA_1/A_2$ (BD Biosciences, San Diego, USA) diluted 1:1000 and, after washing the membranes were incubated for 1 h with $^{125}$I-labeled rabbit anti-mouse IgG (Perkin Elmer, Massachusetts, USA) diluted 1:1000 and visualized by autoradiography (Kodak XOMAT film; Kodak, Heidelberg, Germany).

The wheat allergen 1-Cys-peroxiredoxin was detected in nitrocellulose-blotted wheat fractions by incubating the blots with 1:1000 diluted specific rabbit antibodies or the pre-immune rabbit Ig for control purposes (Pahr et al. 2012). Bound rabbit antibodies were detected with $^{125}$I-labeled anti-rabbit IgG antibodies (Perkin Elmer, Massachusetts, USA) and visualized by autoradiography.

## Enzymatic digestion of gliadin sub-fractions/protein bands and mass spectrometry

One mg/ml concentration of protein from each of the gliadin sub-fractions were dialysed against 0.1 N HCl and 1 mol/L urea pH 2.0 and then digested with pepsin (Sigma, St. Louis, MO, USA) using an enzyme: substrate (w/w) ratio of 1:100 at 37 °C for 2 h. The pH was adjusted to 8.0 with 2.0 N NaOH and the peptic digest of gliadin was further digested with trypsin (Sigma, St. Louis, MO, USA), at 37 °C for 2 h. Trypsin was heat inactivated at 95 °C for 5 min.

Ten µg of the FT DEAE sub-fraction was separated by (10 % w/v) SDS-PAGE and the protein bands were visualized by staining with Coomassie Brilliant Blue R-250. The individual protein bands of the FT DEAE sub-fraction were cut from the gel and digested with trypsin using Trypsin Profile IGD kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions.

The peptides generated were subjected to mass spectrometric analysis by Nano LC–ESI MS/MS. Data were acquired using an UltiMate 3000 $^{TM}$ (Dionex, Vienna, Austria) Nano- LC system coupled to Bruker HCT Ultra ESI trap (Bruker Daltonics, Bremen, Germany) and an online nanospray source. The digested samples were diluted in 5 % acetonitrile in 0.1 % formic acid and separated on a C 8 PepMap 100 column (3 µm, 100 A) (LC Packings, Dionex, Sunnyvale, CA, USA) using a binary

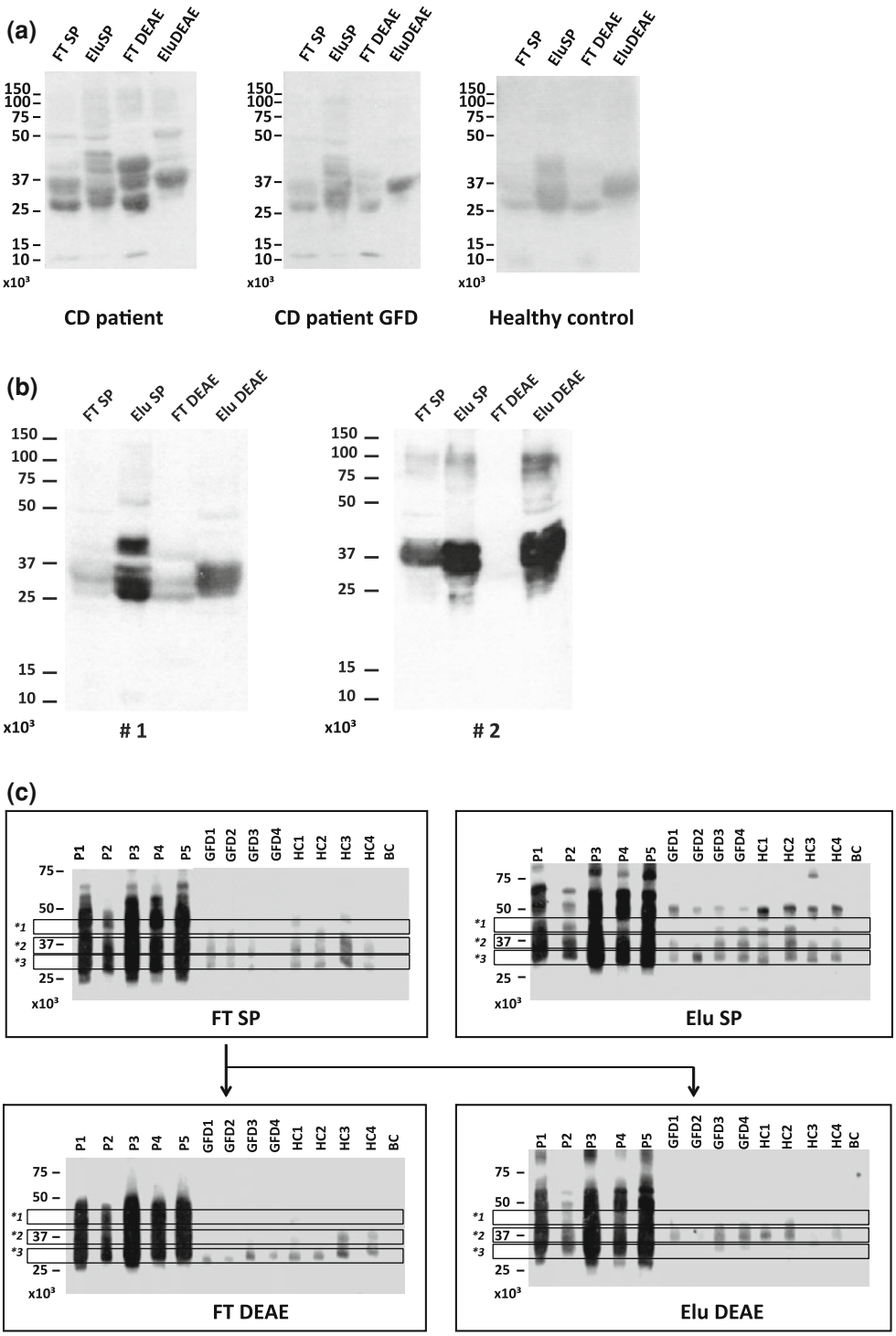



**Fig. 2** IgA reactivity of gliadin sub-fractions. **a** Nitrocellulose-blotted gliadin sub-fractions were probed with serum from a CD patient on a gluten-containing diet (*left panel*), serum from the same CD patient on GFD (*middle panel*) and serum from a control individual without CD (*right panel*). **b** Identically prepared blots exposed to sera from two false AGA positive individuals (#1, #2). **c** Nitrocellulose strips containing blotted gliadin sub-fractions (FT SP, Elu SP, FT DEAE, and Elu DEAE) were probed with sera from CD patients on a gluten-containing diet (*Lanes* P1–P5) and CD patients on GFD (*Lanes* GFD 1–4), control individuals without CD (*Lanes* HC 1–4) or buffer control (*Lanes* BC). Molecular masses $\times 10^3$ of the protein standard are shown on the *left margins*

solvent gradient from 5 to 80 % acetonitrile in 0.1 % formic acid at a flow rate of 300 nL/min over 35-min time interval with a spray voltage of 2.1 kV. During elution, the mass spectra were continuously recorded in a mass/charge range from 300 to 3,000 Da. The three most intense ions were selected for collision-induced dissociation in descending order of signal intensity. Previously found compounds in the selected range were excluded. Data were processed using DataAnalysis$^{TM}$ 3.4 (Bruker Daltonics, Bremen, Germany) where the MS/MS spectra were extracted from the LC-autoMS/MS data set and the compound spectra were then deconvoluted and exported as *.mgf file (MASCOT GENERIC FILE) and the peak list generated was searched against the NCBI and Swiss-Prot

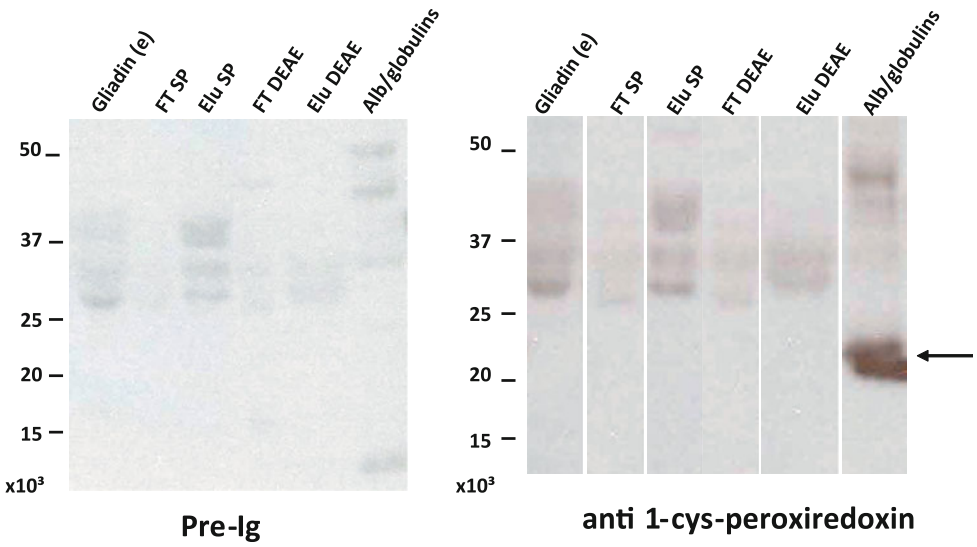



**Fig. 3** Antibody reactivity of nitrocellulose-blotted gliadin fraction, gliadin sub-fractions and the albumin/globulin fraction. Demonstration of successful separation of gliadins and albumins/globulins. Blotted gliadin fraction, gliadin sub-fractions, and the albumin/globulin fraction were probed with Pre-Ig (*left panel*) or rabbit anti-1-Cys-peroxiredoxin antibodies (*right panel*). Molecular masses $\times 10^3$ of the protein standard are shown on the *left margins*

(2012) database subset of plant proteins using the MASCOT (http://www.matrixscience.com) search engine. The search setting included a maximum missed cleavage site value of three, a mass tolerance of ±2 Da for peptide tolerance and ±1 Da for MS/MS tolerance, charges +1, +2 and +3, monoisotopic, variable oxidation of methionine, histidine and tryptophan, and instrument ESI-TRAP. We could not search our data with reversed gliadin database as decoy because of the presence of Proline and Glutamine repeats and lack of variety in the amino acid composition of gliadins (Helmerhorst et al. 2010). For positive protein identification significant MOWSE scores ($p < 0.05$) were employed. Each sample was analysed in duplicates.

### N-terminal sequencing

The FT DEAE sub-fraction was separated by SDS-PAGE (10 % w/v) and electroblotted onto a polyvinylidene fluoride membrane (Immobilon-P, Millipore, MA) pre-wetted with 100 % methanol (Laemmli 1970; Towbin et al. 1979). The membrane was stained with 0.1 % w/v Coomassie brilliant blue R-250 in 50 % v/v methanol, 1 % v/v acetic acid for 30 min and destained with 50 % v/v methanol, 1 % v/v acetic acid to visualize the protein bands. After destaining, the membrane was rinsed in deionised water and air-dried. Bands were excised from the membrane and their N-terminal amino acid sequence was deduced by Edman degradation on an Applied Biosystems Procise 491 sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's chemistry version 1.1.1. The identity of the proteins was determined by comparing the sequence data with the NCBI protein database using the BLAST program (blast.ncbi.nlm.nih.gov).

## Results

### Sub-fractionation of the alcohol-soluble gliadin fraction by ion exchange chromatography

It is well established that the alcoholic fraction of wheat containing gliadins is enriched in antigens which are recognized by IgA antibodies from CD patients. Gliadins are not soluble in aqueous solutions and are composed of a variety of proteins with similar biochemical properties. With the goal to obtain sub-fractions which are enriched in antigens specifically recognized by serum IgA from CD patients, we developed a method using IEC to prepare gliadin sub-fractions with distinct and reproducible protein compositions. The sub-fractionation process involved two consecutive steps of IEC, the first performed with a SP resin and the second with a DEAE resin (Fig. 1a). Gliadin extract (e) was dialysed in Tris buffer containing 2 mol/L urea to remove alcohol and to keep the gliadins in soluble form. When testing different pH conditions we found that for the SP resin, gliadins could be bound at pH 4.5 and eluted with a NaCl gradient. For the DEAE resin pH 10.0 was found to be the best for binding and separation (data not shown). When the FT and elution fractions of the SP column were analysed by SDS-PAGE, we found that compared to the crude gliadin extract (e) bands of a molecular mass of approximately 37,000 and 45,000 were enriched, and the bands at molecular mass of 70,000–75,000 were depleted in the FT SP sub-fraction (Fig. 1b, lanes e, FT SP). The elution SP sub-fractions were a complex mixture of proteins of a molecular mass of 27,000–75,000 (Fig. 1b, lane Elu SP). Next, we fractionated the FT fraction of SP with a DEAE resin and found that protein bands at a molecular mass of 27,000, a 37,000

and 45,000 were further enriched in the FT fraction (Fig. 1b, lanes FT DEAE). The elution fractions of the DEAE column contained a protein band at molecular mass of 55,000 which was clearly separated from the FT and bands between molecular mass of 30,000–45,000 (Fig. 1b, lane Elu DEAE). Attempts were made to fractionate the Elu SP fraction using a DEAE resin, but no further separation of proteins was obtained (data not shown). The described sub-fractionation protocol was repeated several times yielding the four sub-fractions (FT SP, Elu SP, FT DEAE, Elu DEAE) in a reproducible manner, when starting with 25 mg of total protein, approximately 5 mg per sub-fraction were obtained (data not shown).

## IgA-reactive moieties specifically recognized by CD patients are enriched in the FT DEAE sub-fraction

In the next step, we tested the four gliadin sub-fractions for IgA reactivity using sera from CD patients and various controls. Figure 2 shows a representative result obtained with the serum from a CD patient before and after GFD (81 months) and with the serum from a control individual without CD. The left panel of Fig. 2a shows the IgA-reactive bands recognized when the CD patient was on a gluten-containing diet. At that time, the patient showed prominent IgA reactivity to bands with a molecular mass of approximately 27,000 and 37,000 in the FT SP sub-fraction and to an additional band with molecular mass of approximately 45,000 in the FT DEAE sub-fraction (Fig. 2a). IgA reactivity to the bands with a molecular mass of 37,000 and 45,000 in the FT DEAE sub-fraction was markedly reduced when the patient followed a GFD. By contrast, a band with molecular mass of 37,000 which was enriched in the Elu DEAE sub-fraction showed comparable IgA reactivity regardless whether the patient was on gluten-containing or GFD. This band was also recognized by serum IgA from a control individual without CD (Fig. 2a, right panel). Likewise, the band at molecular mass of approximately 27,000 in the FT SP, Elu SP and FT DEAE sub-fractions showed IgA reactivity when the CD patient was on GFD and this band was also recognized by the control individual without CD. Thus, sub-fraction FT DEAE was enriched for CD-specific, IgA-reactive antigens with a molecular mass of approximately of 37,000 and 45,000. By contrast, sub-fraction Elu SP contained IgA-reactive bands with molecular masses ranging from 27,000 to 50,000 which were also recognized by the control individual without CD and the CD patient on GFD. Importantly, the protein band with a molecular mass of 37,000 could be separated into a band specifically recognized by IgA from the CD patient on gluten-containing diet in the FT DEAE sub-fraction and a band of similar molecular mass in the Elu DEAE sub-fraction which was not specific for CD (Fig. 2a).

When we tested sera from two individuals with false positive AGA test results for IgA reactivity to the sub-fractions, we found a similar albeit stronger IgA reactivity profile as compared to healthy persons without CD (Fig. 2b). Both individuals showed pronounced IgA reactivity to the Elu SP and Elu DEAE sub-fractions, whereas they showed weak or almost no detectable IgA reactivity to the FT DEAE sub-fraction further indicating that the FT DEAE sub-fraction was enriched for CD-specific antigens.

We next tested sera from additional CD patients on gluten-containing diet and on GFD and sera from control individuals without CD for IgA reactivity with the four sub-fractions and obtained comparable results. Figure 2c shows representative results obtained with sera from five freshly diagnosed CD patients (P1–P5), two CD patients with different durations of GFD (i.e., patient 1: GFD1–32 months; GFD2–81 months, patient 2: GFD3–4 months; GFD4–18 months) and four healthy controls (HC1–HC4) without CD. For ease of comparison, the regions comprising the molecular mass of 45,000, 37,000 and 25,000 are boxed in the four sub-fractions as **1*, **2*, and **3* respectively (Fig. 2c). Again we observed that the CD-specific antigens with a molecular mass of 37,000 and 45,000 were enriched in the FT DEAE fraction, whereas the Elu SP sub-fraction contained several bands in the molecular mass range between 27,000 and 50,000 recognized by control individuals without CD and CD patients on GFD. A 37,000 molecular mass band which was non-specific for CD as it was detected by the CD patients on GFD and the control individuals (Figs. 2, 3: GFD1–4, HC1–4) was reduced in the FT DEAE sub-fraction and enriched in the Elu DEAE sub-fraction. It has thus been possible to separate by ion exchange IgA-reactive antigens of similar molecular mass (i.e., 37,000 and 45,000) of which certain were specific for CD (FT DEAE sub-fractions), whereas others in the Elu SP and Elu DEAE sub-fractions were also recognized by CD patients on GFD and individuals without CD.

In order to test whether extraction and sub-fractionation methods indeed enrich gliadins and separates them from other wheat proteins, we used antibodies raised against 1-Cys-peroxiredoxin, a recently discovered wheat allergen present in the aqueous-soluble albumin/globulin fraction (Pahr et al. 2012). We found that this allergen was specifically recognized at a molecular mass of approximately 22,000 in the aqueous-soluble wheat protein fraction and not in the gliadin fractions or any of the four sub-fractions (Fig. 3, right panel).

## Identification of the CD-specific bands with a molecular mass of 37,000 and 45,000 in the FT DEAE sub-fraction as γ-gliadins

In a next set of experiments, we further characterized the IgA-reactive antigens in the sub-fractions. In a first

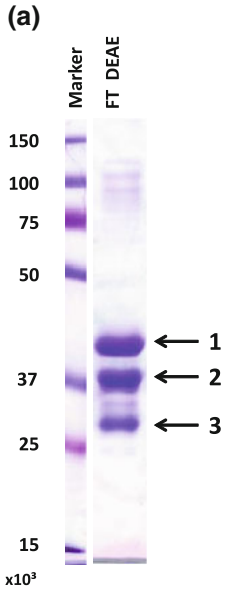
**Table 1** List of peptides identified by LC–ESI–MS/MS after peptic-tryptic digestion of each gliadin sub-fraction

| Fraction | Gliadin | Acc. no. | SC (%) | Sequences identified by ESI–MS/MS | Position | Δ | M | Mascot score | Proteins matching the same set of peptides |
|---|---|---|---|---|---|---|---|---|---|
| FT SP | γ-Gliadin | P06659 | 9 | VPLSQQQQVGQGIL | 223–236 | −0.87 | 1 | 114 | B6UKN4;B6UKP2;Q6EEW6 |
| | | | | VQGQGIIQPQQPAQL | 237–251 | −0.98 | 0 | | |
| | γ-Gliadin | P08453 | 3 | APFASIVAGIGGQ | 315–327 | 0.03 | 0 | 89 | BAA11251;AAF42989;AAK84778 |
| | γ-Gliadin | AAD30556 | 4 | APFSSVVAGIGGQ | 286–298 | −0.09 | 0 | 65 | ACI04108;ACJ03465;ACJ03452 |
| | γ-Gliadin | AAK84779 | 8 | PQQQRPFIQPSL | 166–177 | −0.83 | 1 | 131 | AAQ63858;AAF42989;AED99848 |
| | | | | LPLSQQQQVGQGSL | 259–272 | −0.76 | 2 | | |
| | γ-Gliadin | ACI04109 | 15 | PQQPQQPFPQPQQPQQLF | 78–95 | 1.23 | 2 | 90 | – |
| | | | | YQQQQVGQGTL | 218–228 | −0.66 | 0 | | |
| | | | | VQGQGIIQPQQPAQL | 229–243 | −0.96 | 0 | | |
| | γ-Gliadin | CAC94869 | 9 | PLYQQQQVGQGTL | 210–222 | −0.78 | 1 | 113 | AAD30556;CAC11055; CAC11056 |
| | | | | VQGQGIIQPQQPAQL | 223–237 | −0.98 | 0 | | |
| | α/β-Gliadin | CAY54134 | 6 | QQPNIAHASSQVSQQSYQL | 125–143 | −1.33 | 0 | 62 | BAM08453;BAM08458;BAM08459 |
| | α/β-Gliadin | P04725 | 9 | QQHNIAHASSQVL | 158–170 | −0.64 | 0 | 82 | P04725;AAA34275 |
| | | | | QQQQQQQQQQPSSQVSF | 232–248 | −0.72 | 0 | | |
| E SP | α-Gliadin | AFQ13474 | 5 | GSVQPQQLPQFEEIR | 240–254 | −0.27 | 0 | 76 | – |
| | LMW glutenin | ACP27638 | 6 | AIVYSIILQQQQQQQQQQQQR | 192–212 | −0.49 | 0 | 68 | ABX84154;ACY08811 |
| | γ-Gliadin | ACI04080 | 9 | RPLFQLVQGQGIIQPQQPAQLEVIR | 208–232 | 0.96 | 0 | 66 | ACX37111;ABD98797;ACJ03454 |
| | Avenin-like b1 | Q2A783 | 10 | QQPQQQWQGMYQPQQPAQHESIR | 225–248 | −1.83 | 0 | 97 | P0CZ05;Q2A783 |
| | | | | MSLQALR | 249–255 | −0.54 | 0 | | |
| | α-Gliadin | CAB76956 | 4 | QQHNIAHGSSQVL | 146–158 | −1.08 | 0 | 63 | AFQ13466;P04722;ABQ52119 |
| | α-Gliadin | BAM08452 | 5 | DVVLQQPNIAHASSK | 144–158 | −1.26 | 0 | 91 | BAM08454 |
| | LMW glutenin | ABC84366 | 7 | PQQPPFSQQQQPIL | 150–163 | −0.98 | 1 | 92 | AEI00680;ABY58133;AEI00677 |
| | | | | AQGTFLQPHQIAQL | 302–315 | −0.94 | 2 | | |
| | α-Gliadin | CAB76958 | 10 | VRVPVPQL | 2–9 | −0.46 | 0 | 88 | CAB76959; CAB76963 |
| | | | | RPSQQNPQAQGSVQPQQLPQF | 220 –240 | −1.27 | 1 | | |
| | α-Gliadin | CAB76954 | 10 | VRVPVPQL | 2–9 | −0.70 | 0 | 98 | CAB76957 |
| | | | | QQHNIAHASSQVLQQSSYQQL | 127–147 | −1.54 | 1 | | |
| | α-Gliadin | P04722 | 14 | QQHNIAHGSSQVLQESTYQL | 149–168 | −1.48 | 1 | 75 | AFB35199; AFX69619;AFQ13471 |
| | | | | FQPSQQNPQAQGSFQPQQLPQF | 236–257 | −1.37 | 3 | | |
| | LMW glutenin | ABG45899 | 7 | VQQQIPVVQPSIL | 106–118 | −0.77 | 0 | 62 | – |
| | | | | LQPHQISQL | 248–256 | −0.82 | 1 | | |
| | LMW glutenin | ABC84367 | 11 | PQQPPFSQQQQPIL | 150–163 | −0.98 | 1 | 148 | ABY58133;ACY08822 |
| | | | | PQQQIPFVHPSIL | 179–191 | −0.88 | 1 | | |
| | | | | AQGTFLQPHQIAQL | 302–315 | −0.94 | 2 | | |
| | α/β-Gliadin | P04724 | 6 | VPVPQLQPQNPSQQQPQK | 23–40 | −0.64 | 0 | 46 | – |
| | γ-Gliadin | AAK84773 | 6 | VQGQGIIQPQQPAQL | 202–216 | −0.98 | 0 | 49 | AAK84774;ABD98797 |
| | LMW glutenin | ABY58130 | 9 | LQQQIPIVHPSIL | 113–125 | −0.89 | 1 | 92 | – |
| | | | | AQGTFLQPHQIAQL | 236–249 | −0.94 | 2 | | |

**Table 1** continued

| Fraction | Gliadin | Acc. no. | SC (%) | Sequences identified by ESI–MS/MS | Position | Δ | M | Mascot score | Proteins matching the same set of peptides |
|---|---|---|---|---|---|---|---|---|---|
| FT DEAE | γ-Gliadin | ACJ03413 | 6 | PFIQPSLQQR | 190–199 | 0.06 | 0 | 120 | ACJ03424;ACJ03436;ACJ03435 |
| | | | | APFASIVAGIGGQ | 336–348 | 0.65 | 0 | | |
| | γ-Gliadin | A56891 | 100 | NIQVDPSGQVQAL | 1–13 | 0.58 | 0 | 44 | – |
| | γ-Gliadin | ACI04082 | 9 | NIQVDPSGQVQWLQQ | 2–16 | −1.54 | 1 | 74 | P08453 |
| | | | | VQGQGIIQPQQPAQL | 255–269 | −1.44 | 0 | | |
| | γ-Gliadin | P08079 | 7 | AQIPQQL | 195–201 | −0.41 | 0 | 70 | ACJ03452;CAC11055;CAC94868 |
| | | | | YQQQQVGQGTL | 233–244 | −0.66 | 0 | | |
| E DEAE | γ-Gliadin | CAC94869 | 10 | PLYQQQQVGQGTL | 210–222 | −0.98 | 1 | 152 | AAD30556;CAC11055;CAC11056 |
| | | | | VQGQGIIQPQQPAQL | 223–237 | −1.04 | 0 | | |
| | γ-Gliadin | AAF42989 | 3 | APFASIVAGIGGQ | 296–308 | −0061 | 0 | 106 | AAK84778;AAK84779; ACI04081 |
| | α-Gliadin | AFQ13474 | 5 | GSVQPQQLPQFEEIR | 240–254 | −1.01 | 0 | 69 | ADM96155;ACJ76946 |
| | α-Gliadin | BAA12318 | 19 | QQQQPSSQVSFQQPLQQYPLGQGSFR | 180–205 | −0.65 | 0 | 142 | AFQ13475;BAM08462;ACJ76944 |
| | | | | PSQQNPQDQGSVQPQQLPQFEEIR | 206–229 | −1.58 | 0 | | |
| | α-Gliadin | ADM96162 | 12 | QQHSIAHGSSQVLQQSTYQL | 159–178 | −1.42 | 1 | 102 | – |
| | | | | HHQQQQQQQQPPLSQVF | 209–225 | −0.40 | 1 | | |
| | α/β-Gliadin | CAB76964 | 7 | VRVPVPQL | 1–8 | −0.66 | 0 | 102 | – |
| | | | | QQHSIAYGSSQVL | 150–162 | −0.97 | 0 | | |
| | α/β-Gliadin | BAM08455 | 10 | QQQQQQQQQQIL | 117–129 | −1.88 | 0 | 96 | BAM08456 |
| | | | | QQPNIAHASSQVSQQSYQL | 146–164 | −1.37 | 0 | | |
| | γ-Gliadin | ACJ76949 | 4 | VQGQGIIQPQQPAKL | 257–271 | −1.08 | 0 | 69 | – |

*Acc.no* NCBI/Swiss−Prot accession number, *SC* sequence coverage, *Δ* difference between the measured peptide mass Mr (expt) and theoretical mass of the peptide Mr (calc), *M* missed cleavages, *LMW* low molecular weight




(b)

| Bands | Peptide identified after tryptic in-gel digestion and ESI-LC MS/MS | N-terminal sequence |
|---|---|---|
| 1 | APFASIVAGIGGQ (γ- gliadins) | NMQVDPSGQVQQ (γ- gliadins) |
| 2 | APFASIVAGIGGQ (γ- gliadins) | NIQVDPSGQ (γ- gliadins) |
| 3 | APFASIVAGIGGQ (γ- gliadins) | NIQVDPSSQ (γ- gliadins) |

**Fig. 4** Mass spectrometry and N-terminal sequencing of individual protein bands in the FT DEAE sub-fraction. SDS PAGE **a** containing three major bands of the FT DEAE sub-fraction are marked (1–3). The table **b** lists sequences of peptides which were identified by mass spectrometry following a trypsin in-gel digestion and by N-terminal sequencing of the three bands

screening approach, the sub-fractions were digested by pepsin and trypsin using an in-solution method and the peptides were then separated by RP–HPLC and analysed by LC–ESI–MS/MS. The analysis of the most frequent peptide sequences revealed that the FT DEAE sub-fraction containing CD-specific antigens was mainly composed of γ-gliadins, whereas the elution SP fraction reacting also to IgA from individuals without CD contained primarily α/β gliadins and low molecular weight (LMW) glutenins. In fact, it has been described already earlier that glutenins may co-purify with gliadins (Huebner and Bietz 1993). The Elu DEAE sub-fraction contained a mixture of γ-gliadins and α/β-gliadins (Table 1).

Next, we prepared a FT DEAE sub-fraction for LC–ESI–MS/MS analysis and N-terminal sequencing. The Coomassie-stained FT DEAE (Fig. 4a) sub-fraction contained three prominent bands of a molecular mass of 27,000, 37,000 and 45,000 which were excised and subjected to tryptic in-gel digestion and subsequent LC–ESI–MS/MS. The blotted bands were also subjected to N-terminal sequencing. Results taken together from the tryptic digestion and subsequent LC–ESI–MS/MS and the N-terminal sequencing indicated that the three proteins in the FT DEAE fractions were derived from different γ-gliadins (Fig. 4b).

## Discussion

Identifying CD-specific wheat antigens has been hampered by the great variety of gliadins and their physicochemical properties which make their separation difficult. Here, we report a reproducible immuno-proteomics approach for sub-fractionating the gliadin fraction which allowed enriching of antigens specifically recognized by IgA antibodies from CD patients with active disease.

By applying two steps of ion exchange chromatography, we could enrich CD-specific antigens in the final FT DEAE sub-fraction. Interestingly, we found in other sub-fractions and in particular in the Elu SP sub-fraction wheat antigens which also reacted with IgA antibodies from individuals without CD. These antigens had a similar molecular weight as the proteins in FT DEAE sub-fraction which were specifically recognized by CD patients, but differed regarding their chromatographical behavior. The analysis of the digested sub-fractions by LC–ESI–MS/MS revealed that α/β-gliadins as well as LMW-glutenins were enriched in the sub-fractions Elu SP and Elu DEAE suggesting that these antigens may be less specific for CD (Table 1). This result is interesting in light of the fact that several studies emphasize the specificity and the importance of α-gliadins for CD (Anderson et al. 2000; Shan et al. 2002; Bateman et al. 2004). Our study rather highlights the importance of γ-gliadins for CD, at least at the level of antibody recognition. MS/MS analysis as well as N-terminal sequencing revealed that FT DEAE sub-fraction contained certain γ-gliadins of a molecular mass of 37,000 and 45,000 which appeared to be specific for CD (Table 1; Fig. 4). The finding that CD patients on GFD lost IgA reactivity to the γ-gliadins with molecular mass of 37,000 and 45,000 in the FT DEAE fraction, but retained IgA reactivity to antigens in the Elu SP and Elu DEAE fractions further confirmed that the purification protocol enriched γ-gliadins as CD-specific antigens.

One reason why several studies emphasize the importance of α-gliadins for CD, whereas we found γ-gliadins as specific

targets in CD is that in most studies either T cell or antibody reactivity is used as parameter for immunoreactivity.

Although LMW-glutenins, a subunit of glutenins are related to gliadins and have similar amino acid sequence and domain arrangement (Wieser 2007), our sub-fractionation protocol was able to separate the LMW-glutenins from γ-gliadins (Table 1) (Huebner and Bietz 1993). The IgA reactivity of the individuals without CD against the proteins in Elu SP and Elu DEAE may, thus, be a sign of normal exposure to wheat which is similar to the development of IgG and IgA antibodies against food antigens (e.g., milk antigens) in healthy persons (Hochwallner et al. 2011). The IgA reactivity towards α-, β-gliadin and glutenin-rich sub-fraction in the CD patients on diet may be explained by exposure to food which does not cause CD, but contains cross-reactive antigens which show sequence similarity with α-, β-gliadins as well as glutenins, but not to γ-gliadins with a molecular mass of 37,000 and 45,000 in the FT DEAE sub-fraction.

In fact, it is also known that individuals without CD exhibit IgA antibody reactivity to the crude gliadin fraction (Skerritt et al. 1987; Constantin et al. 2005). The presence of antigens in the gliadin fraction also recognized by IgA from individuals without CD may also explain why AGA-based diagnostic tests frequently show false positive results causing poor specificity and are, therefore, being discontinued in many diagnostic laboratories (Rostom et al. 2005). The results from our study provide evidence that crude gliadin extracts indeed contain moieties which are not specific for CD. In fact, we found that sera from individuals with false positive AGA tests reacted with the Elu SP and Elu DEAE sub-fractions, but not with the FT DEAE sub-fraction which was enriched for CD-specific antigens. Therefore, the γ-gliadins with a molecular mass of 37,000 and 45,000 from the FT DEAE sub-fraction may be used to increase the specificity of serological tests for CD.

There is also a particular need for serological tests monitoring adherence to GFD or intestinal recovery (Leffler and Schuppan 2010). In this context, we found that the FT DEAE fraction which contained γ-gliadins indeed showed much lower IgA reactivity with sera from individuals without CD or from CD patients on GFD when used for IgA testing than the other sub-fractions. It is therefore possible that the CD-specific γ-gliadins can be used for serological monitoring of the adherence to GFD.

In summary, we think that our results may contribute to the elucidation of pathogenic epitopes and mechanisms in CD and for the improvement of diagnostic tests for CD.

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**Conflict of interest** Rudolf Valenta serves as a consultant for Phadia (Thermofisher). The rest of the authors have no conflict of interest to declare.

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