



Wheat-induced food allergy in childhood: ancient grains seem no way out

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Abstract

Purpose Wheat is a frequent elicitor of food allergy in childhood. Especially in popular media, the better digestibility and the lower allergenicity of ancient grains are repeatedly postulated. We addressed the question whether ancient wheat-related grains are less allergenic than modern wheat.

Methods Proteins from cultivars of spelt, einkorn, emmer and durum along with durum soft wheat flour, Triticum and bread wheat were separated by electrophoresis. Immunoblots were performed with a pool serum of six sera from wheat-sensitized children aged 1–11 years (wheat-specific IgE 22 kU_A/l). As controls, pool serum from five sera atopic patients aged 3–13 years who had no sensitization to wheat (wheat-specific IgE 0.11 kU_A/l) and six sera from non-atopics at the age of 3 months to 5 years (wheat-specific IgE 0.06 kU_A/l) was used. Area under the curve (AUC) in Coomassie-stained gels and immunoblots was determined and related.

Results Water/salt-soluble protein patterns were very similar among varieties. In einkorn cultivars, one protein band corresponding to an alpha-amylase/trypsin inhibitor (ATI) was absent. Water-insoluble protein fractions differed markedly among varieties and cultivars. IgE bound to a large number of proteins in all protein fractions both in wheat and in the wheat-related ancient grains.

Conclusions Patients with sensitization to wheat show a significant IgE binding against both modern and ancient grain varieties of the genus Triticum. Therefore, ancient grains do not appear to have a generally reduced risk for wheat allergy sufferers. However, few individuals only sensitized to ATI could benefit from the consumption of einkorn.

Keywords Wheat allergy · Ancient grains · ATI · IgE · Triticum

Introduction

Wheat is, along with maize and rice, one of the three staple crops, providing micronutrients and a significant energy uptake in daily diet, especially in bakery products (*Triticum aestivum* L., soft bread wheat) and pasta (*Triticum turgidum* ssp. *durum* (Desf.) Husn, hard wheat). Due to its unique

viscoelastic properties, wheat gluten is also an important thickener and filler in food industry and thereby hard to be eliminated from daily diet [1, 2]. In 2017, 771 million tons were produced worldwide (source FAOSTAT 2018 <http://www.fao.org/faostat/en/#data/QC>), and it is assumed that the global demand for wheat will further increase. On the other hand, wheat allergy is a common disease, and allergic individuals or their caregivers ask for alternative “ancient” cereals [einkorn (*Triticum monococcum* L.), emmer (*Triticum turgidum* ssp. *dicoccum* (Schränk ex Schübl.) Thell.), Khorasan (*Triticum turanicum* Jakubz.), rivet (*Triticum turgidum* L.), and spelt (*Triticum aestivum* ssp. *spelta* (L.) Thell.)] which are not only believed to be less allergenic but also of nutritional benefit and ecologically more sustainable than highly cultivated “modern” bread wheat [3–6]. Comprehensive reviews of recent studies on nutritional aspects have been provided by Dinu et al. [7] or Shewry et al. [8].

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Family	Subfamily	Supertribe	Tribe	Sub-Tribe	Genus	Name(s) of members
Poaceae	Oryzoideae		Oryzeae	Oryzinae	Oryza	Rice
	Pooideae	Poodae	Poeae	Avenineae	Avena	Oat
		Triticoideae	Triticeae	Hordeinae	Hordeum Secale	Barley Rye
				Triticinae	Aegilops	Goatgrass
					Triticum	Wheat, Spelt, Emmer, Einkorn
	Panicoideae	Andropogonodae	Andropogoneae	Tripsacinae	Zea	Maize
				Sorghinae	Sorghum	Sorghum

Fig. 1 Phylogenetic relations between grains most relevant in nutrition. Derived and modified from [77]

Next to celiac disease, which is a T cell-mediated chronic inflammatory enteropathy in genetically pre-disposed individuals, immunoglobulin E (IgE)-mediated wheat allergy affects up to 0.5% of western pediatric and 1% of the adult population [9–11]. Especially in adults, augmentation (risk) factors such as physical activity, drugs or alcohol play an important role. Wheat-dependent exercise-induced anaphylaxis (WDEIA) is a well-known disease resulting in anaphylaxis in adults [12]. Moreover, wheat protein can also cause occupational asthma with a prevalence of 4% to 25% among bakers [13, 14]. In usually tolerant individuals, processing, especially thermal treatment, may also alter gluten proteins (de-amidation of glutamine to glutamate) which can cause allergic reactions [15]. Recently, non-celiac gluten/wheat sensitivity (NCGWS) has been suggested as clinical picture [16], triggered by alpha-amylase/trypsin inhibitors (ATI), but without involvement of IgE. Due to missing clear diagnostic criteria and clinical validation, NCGWS is questioned by most allergists [17]. Another disease without clear serological markers, the irritable bowel syndrome (IBS), causes similar symptoms, and the relation to NCGWS is still in discussion [18].

The claimed increase in wheat-related diseases is especially attributed to the augmented consumption of genetically homogeneous “modern wheat”, originating from the beginning of the twentieth century when breeding focused on the improvement of gluten quality and reduced height [19], cultivation under pesticides, the consumption in highly processed form (usually not as whole grain like “ancient grains” with rich content of secondary plant compounds), viral infections [20] and perceptual awareness of customers. Recent research has focused on celiac disease due to the fact that the pathogenesis is well known and that

standardized test systems (Codex Alimentarius standard R5 monoclonal antibody [21]) for the detection of toxic epitopes of gluten proteins are available, although non-gluten proteins have been suggested to be also involved [22]. The role of breeding on wheat toxicity and the benefit of ancient grains, especially einkorn, has been a matter of considerable debate [23–29]. In contrast to celiac disease, there are few studies investigating beneficial effects of ancient grains for patients suffering from IgE-mediated wheat-related disorders [30–32].

So far, more than 40 allergenic components in wheat have been described (www.allergome.org), and usually, IgE of sensitized individuals binds to a broad spectrum of proteins [33]. The majority of them belong to the water- and salt-soluble albumin/globulin fraction (15–20% of total protein content) which comprises enzymes and defense proteins. The water-insoluble protein fraction (gluten, 80% of total protein content) consists of monomeric gliadins [specified as α/β -, γ - and ω -gliadin according to their electrophoretic separation in acidic polyacryl gel electrophoresis (PAGE)], polymeric high molecular weight (HMW) and low molecular weight (LMW) glutenins. Water-insoluble storage proteins are also found in other grains such as in rye (secalin), barley (hordein) and oats (avenin).

Phylogenetically, wheat and so-called ancient grains except oats and sorghum/millet belong to the genus *Triticum* within the Poaceae family (Fig. 1). While ancient wheat varieties are diploid with $2n = 14$ chromosomes such as *T. monococcum* (genome AA), *Aegilops speltoides* (BB) or *Aegilops tauschii* (DD), “modern” varieties are the result of hybridizations at the beginning of the twentieth century when breeding focused on the improvement of gluten quality, and in the 1950s, when dwarfing genes were introduced

Table 1 Characteristics of individual sera used for serum pools

Pool	Sex	Age (months)	fx5 (kU _A /l)	sx1 (kU _A /l)	f4 (kU _A /l)	f4 (kU _A /l) in pool
Non-atopic	w	48	0.04	0.05	n.d.	0.06
	m	20	0.00	0.05	n.d.	
	w	16	0.04	0.04	n.d.	
	m	3	0.05	0.06	n.d.	
	w	63	0.07	0.11	n.d.	
	m	11	0.02	0.09	n.d.	
Atopic	m	90	0.05	22.5	n.d.	0.11
	m	140	0.15	22.8	n.d.	
	m	42	0.14	>100	n.d.	
	w	157	0.19	53.6	n.d.	
	m	89	0.21	4.13	n.d.	
	w	15	18.9	19.1	12.8	22.0
Wheat-sensitized	m	23	n.d.	n.d.	10.4	
	w	42	11.3	17.2	10.4	
	w	133	3.94	> 100	5.06	
	m	17	4.18	n.d.	9.68	
	m	19	> 100	n.d.	69.1	

Specific IgE to wheat (f4), a mixture of 6 food allergens (cow's milk, hen's egg, wheat, peanut, soy and codfish) (fx5) and 7 inhalant allergens (house dust mite, cat and dog dander, mold, timothy grass, mugwort and birch pollen (sx1) was determined using the ImmunoCap system (Thermo Fisher). Results <0.35 kU/l were considered negative. n.d., not determined

to limit height and ease threshing out grains, leading to tetraploid *T. turgidum* ssp. *durum* and hexaploid *T. aestivum* with ancestral genomes AABB and AABBDD, respectively [19]. “Heritage” varieties are such developed before this time, comprising landraces with mixed genotypes. Recent molecular data indicate a complex hybrid speciation of each contributing genome as reviewed in detail by Haberer et al. [34]. The polyploidy of hexaploid wheat results in a genome size with approximately 14.5 Gb and 107,891 high confidence genes plus 161,537 low confidence genes as recently reported by the International Wheat Genome Sequencing Consortium [35].

In this study, we addressed the question whether ancient grains differ significantly from modern wheat in their ability to be recognized by IgE from wheat-sensitized individuals. In order to cover a broad spectrum of protein to IgE interaction, we used pooled serum of six individuals (aged 15 to 133 months) with sensitization to wheat (specific IgE 5.06 to 69.1 kU_A/l) for western blot analyses of albumin/globulin, gliadin and glutenin/residual protein fractions of each two representative cultivars of einkorn, emmer and spelt along with three samples of durum, one modern wheat and a hybrid of barley/wheat (Tritordeum). Results obtained in immunoblot analysis were correlated with findings in Coomassie protein stain and matrix-assisted

laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Additional samples were used to confirm results and to complete the picture of IgE reactivity to representatives of the Pooideae family.

Materials and methods

Sera and detection of specific IgE

Pooled sera of six (age 1–11 years) wheat-sensitized, five (age 3–13 years) atopic and six (age 3 months–5 years) non-atopic individuals were used. Wheat-specific IgE in the pooled sera were 22 kU_A/l, 0.11 kU_A/l and 0.06 kU_A/l, respectively. Details of individual sera are shown in Table 1. Specific IgE to wheat (f4), common childhood food allergens (cow's milk, hen's egg, wheat, peanut, soy and codfish; fx5 panel) and inhalant allergens (house dust mite, cat and dog dander, mold, timothy grass, mugwort and birch pollen; sx1 panel) was determined on ImmunoCap (Thermo Fisher Scientific, Darmstadt, Germany). Results <0.35 kU/l were considered negative. The study was approved by the local institutional review board (ethical approval number EA2/064/13).

Protein extraction, SDS-PAGE and immunoblotting

250 mg flour were diluted in 1.5 ml 0.3 M NaI (or 0.5 M NaCl where indicated) and stirred at room temperature for 1 h. Water- and salt-soluble proteins (albumins/globulins) were obtained by centrifugation at $16,100\times g$ for 5 min. The pellet was crushed and washed each two times with 0.3 M NaI and distilled water. Gliadins were extracted with 1.5 ml 50% propan-2-ol for 1 h under stirring. After washing two times, 1.5 ml lithium dodecyl sulfate (LDS) sample buffer (Thermo Fisher Scientific, Darmstadt, Germany) with 200 mM dithiothreitol (DTT) (Applichem, Darmstadt, Germany) were applied. The suspension was heated at 70 °C for 10 min, stirred for 1 h and centrifuged to obtain glutenins and residual proteins

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D SDS-PAGE) and immunoblotting were performed as described previously [33]. Briefly, equal volumes of water- and salt-soluble proteins in LDS sample buffer with 50 mM DTT and a molecular weight marker (Spectra Multicolor Broad Range Protein Ladder, PageRuler Unstained Broad Range Protein Ladder, Thermo Fisher Scientific, Darmstadt, Germany) were separated in SDS-PAGE on 12% or 4–12% Bis–Tris gels (Thermo Fisher Scientific, Darmstadt, Germany) with 2-(N-morpholino)ethanesulfonic acid (MES) running buffer. Water-insoluble gliadins and glutenins were separated on 12% Bis–Tris gels by extended electrophoresis with 3-(N-morpholino)propanesulfonic acid (MOPS) running buffer. Proteins were blotted onto 0.45 µm Immobilon-P membrane (Merck Millipore, Burlington, Massachusetts, USA) at 30 V for 1 hour. Membranes were incubated for 2 h each with pool serum, diluted 1:5 in PBS–Tween 0.05% (v/v) with 1% bovine serum albumin (BSA) (Sigma-Aldrich) and 10% Goat serum (Thermo Fisher Scientific, Darmstadt, Germany) in the sequence albumins/globulin, gliadins and glutenins/residual proteins. Negative and atopic (no food allergy, but allergy to house dust mite) pool sera were applied accordingly. The background control was only treated with the diluent phosphate buffered saline (PBS)–Tween 0.05% (v/v) with 1% BSA and 10% Goat serum (v/v). After washing three times with PBS, the membranes were incubated with ^{125}I -labeled anti human IgE from goat (Ringel Immunologische Laboratorien, Aachen) in the diluent. Exposition was conducted at -80 °C for 10 and 5 days on Carestream Kodak BioMax MS films (Sigma-Aldrich, St. Louis, Missouri, USA) which were scanned on a GS-800 densitometer (BioRad, Hercules, California, USA). Signals were enhanced for optimal visualization. Reference gels for protein visualization and excision of bands were stained with colloidal Coomassie overnight. Images of gels and x-ray films were analyzed with Quantity One vers. 4.6.9 (BioRad, Hercules,

California, USA) and calculation of area under the curve (AUC) was performed with Prism vers. 5.03 (GraphPad, La Jolla, California, USA).

Statistical analysis

Coomassie-specific AUC, immunoblot-specific AUC and the ratio were log-transformed and submitted to one-way ANOVA with Kruskal–Wallis and Dunn's multiple comparison test statistic (Prism vers. 5.03). A *P* value < 0.05 was considered statistically significant.

MALDI-TOF mass spectrometry

Bands of interest were excised, destained with 25 mM NH_4HCO_3 (Merck, Darmstadt, Germany) in 50% acetonitrile (ACN; VWR, Radnor, Pennsylvania, USA), reduced with 10 mM tris(2-carboxyethyl)phosphine (Merck, Darmstadt, Germany) at 60 °C for 10 min and alkylated with 100 mM iodoacetamid (Merck, Darmstadt, Germany) at room temperature for 1 h. The digestion was conducted with trypsin (Thermo Fisher Scientific, Darmstadt, Germany) at 0.02 mg/ml final concentration at 37 °C overnight. MALDI-TOF MS and data processing were performed with equipment and software of Bruker Daltonik, Bremen, Germany. The proteolytic digests were lyophilized and reconstituted in 0.1% trifluoroacetic acid (TFA; Applichem, Darmstadt, Germany) in 50% ACN. A saturated matrix solution of α -cyano-4-hydroxy cinnamic acid (Sigma-Aldrich) was prepared in 30:70 (v/v) ACN:TFA 0.1%, and equal volumes of sample and matrix solution were mixed and applied onto a polished steel target. The instrument (AUTOFLEX-III LRF 200-CID) was calibrated with the peptide calibration standard II, and the detection range was set to 680 to 5000. Data processing was performed with FlexAnalysis vers. 3.3. Spectra were smoothed and a signal-to-noise ratio of 3 for peak finding was applied. Sequence database search using the monoisotopic *m/z* values of the digested peptides was performed with BioTools vers. 3.2 via MASCOT search program (Matrix Science Ltd., London) in SwissProt database, taxonomy “Other green plants”. For the results presented, parameters during the search were: carbamidomethylation (global modification), mass tolerance MS (peptide mass error) of 100 ppm, and 0 partials (number of missed cleavages). Individual protein scores greater than 55 were considered significant (*P* < 0.05).

Further analysis of the data was performed using the software “Sequence editor” vers. 3.2 For suggested ATI proteins, sequences were retrieved from UniProt database and submitted to a theoretical *in silico* digestion with trypsin without the signal sequence of the respective protein. Recorded *m/z* values of samples were matched with calculated fragments applying carbamidomethylation (global modification) and

Table 2 Area under the curve (AUC) of optical density (OD) determined in Coomassie-stained gels and immunoblots (IB) of albumin/globulin, gliadin and glutenin fraction, and calculation of ratio

Fraction	Variety	Spelt		Einkorn		Emmer		Durum				Wheat
	Cultivar	Oberkul- mer Rotkorn	Franckenkorn	Terzino	Tifi	Osiris	Ramses	Wintergold	Soft wheat	Flour	Tritordeum	Akteur
A/G	Coomassie	0.139	0.137	0.172	0.210	0.174	0.178	0.163	0.209	0.146	0.238	0.206
	Immunoblot	0.213	0.204	0.208	0.227	0.216	0.216	0.215	0.259	0.237	0.269	0.252
	Ratio	0.651	0.668	0.827	0.925	0.806	0.825	0.757	0.808	0.613	0.884	0.815
Gliadin	Coomassie	0.023	0.011	0.024	0.014	0.012	0.009	0.014	0.034	0.021	0.027	0.021
	Immunoblot	0.159	0.125	0.107	0.097	0.138	0.124	0.139	0.144	0.143	0.156	0.149
	Ratio	0.143	0.088	0.229	0.145	0.087	0.075	0.098	0.238	0.150	0.176	0.139
Glutenin	Coomassie	0.051	0.054	0.076	0.065	0.090	0.089	0.087	0.158	0.147	0.137	0.120
	Immunoblot	0.165	0.160	0.173	0.153	0.181	0.186	0.177	0.193	0.199	0.206	0.179
	Ratio	0.307	0.337	0.441	0.424	0.496	0.477	0.493	0.819	0.740	0.666	0.671

Shading specific for each Coomassie, IB and ratio, reflecting height of OD and value, respectively

oxidation of methionine (optional modification). 100 ppm mass tolerance and 1 partial were accepted.

Results

Protein and IgE recognition pattern to albumins and globulins

We compared two cultivars (cv.) of spelt, einkorn, emmer, durum along with durum superfine flour and an elite bread wheat. The protein band pattern in 1D SDS-PAGE ranged from M_r 5 to 150 kDa and showed high similarities between varieties with significant exception of einkorn with least corresponding protein bands. Within varieties, slight differences in the protein band pattern occurred between the two spelt cultivars at approximately M_r 43 kDa, more considerable differences were observed in emmer cultivars at M_r 14.6, 51 and 66 kDa.

The protein content per sample and IgE-reactivity (proteins were extracted with equal volumes) were compared by determination of the area under the curve (AUC) (Table 2). Protein content per volume (concentration) was lowest in spelt cv. Franckenkorn and highest in Tritordeum. Differences between cultivars were lowest among spelt and emmer and highest among einkorn, but statistically not significant.

With the exception of einkorn, the IgE recognition pattern in immunoblot ranged from M_r approximately 13 to 95 kDa and was also characterized by a high degree of similarity between varieties (Fig. 2, top row). Negative, atopic and background controls showed only low unspecific signals (Data not shown). Strongest positive signals were observed in protein ranges between M_r 30 kDa and 65 kDa, and in the range from 12 kDa to 17 kDa. One band at M_r 22.5 kDa was

highly specific for durum soft wheat and Tritordeum (Fig. 2, top row). Einkorn showed a distinctively different pattern between M_r 10 kDa and 25 kDa with no clearly positive signals. We therefore analyzed the protein band range between M_r 5 and 16 kDa with MALDI-TOF MS (see below).

Analysis of IgE reactivity strength in immunoblots (IB) revealed no or only slight differences among the varieties without statistical significance. The difference between lowest immunoreactivity (in spelt cv. Franckenkorn) and the highest (hybrid Tritordeum) was only 0.065 units optical density (OD). The ratio of OD Coomassie/Immunoblot was slightly different among spelt and emmer cultivars, but clearly different among einkorn cultivars. The ratio in durum samples varied considerably by almost 0.2 units OD (soft wheat and flour). Of note, the highest ratio was determined for einkorn cv. Tifi. Altogether, all cultivars of varieties tested were positive in immunoblot and showed binding of IgE to a large set of protein bands. Significant differences in ratios of AUC from Coomassie and IB were not found.

Protein and IgE recognition pattern to gliadins

Gliadins were separated by extended electrophoresis and showed protein bands up to M_r of approximately 123 kDa, typical of high molecular weight glutenins. However, strongest protein bands occurred below M_r 52 kDa, representing γ - and α/β -gliadins. Less strong bands between M_r 52 kDa and 60 kDa represent ω -gliadins which migrate in this range. As shown in the middle row of Fig. 2, the protein band patterns differ clearly between varieties. Differences within varieties were lowest in einkorn and durum cultivars. Spelt cv. Franckenkorn had an additional band at M_r of approximately 38 kDa, and emmer protein pattern differed in two bands at M_r of approximately 39 kDa and 37 kDa.

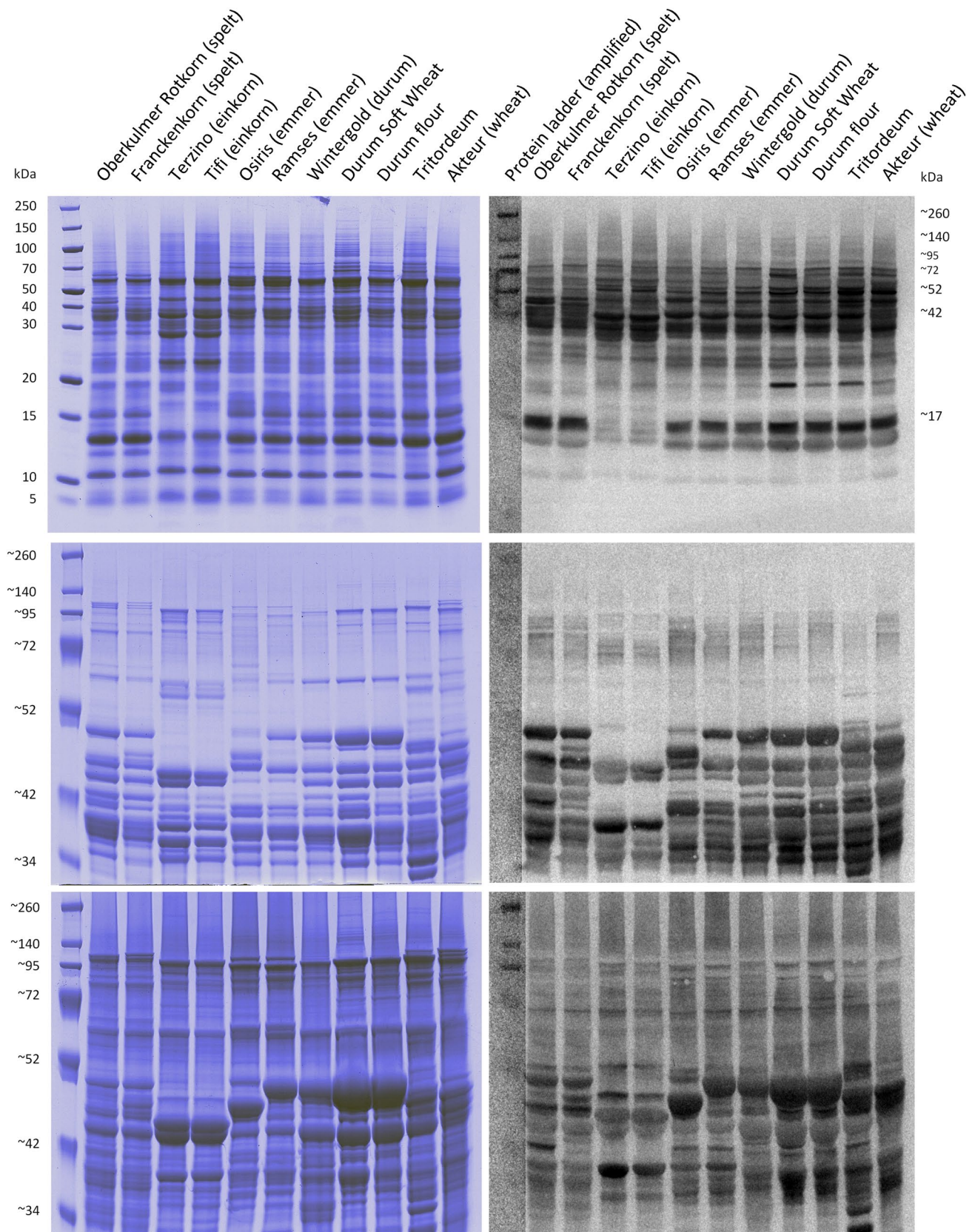


Fig. 2 Proteins separated in 12% Bis-Tris polyacrylamide gels stained with Coomassie (left) and immunoblots with positive pool serum (right) of protein fractions. Top row, water/salt-soluble albumins and globulins middle row: gliadins, bottom row: glutenins and residual proteins

Coomassie staining of gliadins produced lower OD compared to albumin and globulin proteins due to the lower content of basic amino acids (lysine, arginine and histidine) necessary for Coomassie binding. Protein content per volume was lowest in emmer cv. Ramses and highest in durum soft wheat. The least OD difference was observed between emmer cultivars. Within other varieties' cultivars, the OD considerably differed (Table 2, middle row). However, these differences were not statistically significant.

Binding of IgE was detected to almost all protein bands below M_r 52 kDa visible in Coomassie stain. Although contents of high-molecular weight glutenins were visible in Coomassie staining, no clear binding of IgE was observed. Controls showed only low unspecific signals (data not shown).

Reactivity in immunoblots was lowest in emmer cv. Ramses, and highest in spelt cv. Oberkulmer Rotkorn, but the overall difference between these two was low with only 0.062 OD units. The ratio of OD Coomassie/Immunoblot was slightly different only among emmer cultivars, but clearly different among spelt and einkorn cultivars, but most clearly among durum samples, varying by 0.14 units OD (soft wheat and cv. Wintergold). Interestingly, the spelt cultivars differed less in the overall IgE-reactive protein pattern, but more in the band-specific OD which implies qualitative and/or quantitative differences in the interaction with IgE. As observed in the albumin/globulin fraction, all cultivars of varieties were positive in immunoblot and showed binding of IgE to a large set of protein bands despite clear differences in the individual protein pattern. Analysis of IB-specific AUC and ratio of Coomassie/IB revealed no statistically significant differences.

Protein and IgE recognition pattern to glutenins and residual proteins

Glutenins and residual proteins were also separated by extended electrophoresis. The protein pattern showed some similarities to that of gliadins, however, high molecular weight subunits between M_r of approximately 80 and 120 kDa were more prominent, indicating overall extraction of sulfur-containing glutenins. Despite high background in both Coomassie stain and immunoblot, distinctive IgE-specific signals for low molecular weight subunits ($M_r < 52$ kDa) were detected in all extracts.

As shown for gliadins, the protein band patterns differed clearly between varieties (Fig. 2, bottom row). Differences within varieties were lowest in einkorn. Durum cv. Wintergold almost lacked two protein bands at $M_r \sim 80$ kDa and ~ 87 kDa compared to other durum samples. Spelt cv. Franckenkorn had an additional band at $M_r \sim 44$ kDa,

and emmer protein pattern differed in two bands at M_r range ~ 44 kDa to ~ 47 kDa.

Coomassie staining of glutenins and residual proteins produced slightly higher OD compared to gliadins. Protein content per volume was lowest in spelt cv. Franckenkorn and highest in durum soft wheat. Low OD differences were observed among spelt and emmer cultivars, a slight difference was found in cultivars of einkorn. The durum group showed similar OD for soft wheat and flour, and a clearly reduced OD in cv. Wintergold. Reactivity in immunoblots ranged from 0.153 (einkorn cv. Tifi) to 0.206 (Tritordeum). The ratio of OD Coomassie/Immunoblot was quite similar among cultivars of each spelt, einkorn and emmer, but overall differed from 0.307 (spelt cv. Oberkulmer Rotkorn) to 0.819 (durum soft wheat). As for gliadins, clear differences were observed among durum samples, varying by 0.326 units OD (soft wheat and cv. Wintergold). Of note, the two spelt and emmer cultivars considerably differed in protein and IgE-reactive pattern corresponding to differences in protein band pattern. Controls showed low unspecific IgE-binding (Data not shown). In sum, all cultivars of varieties were positive in immunoblot and showed binding of IgE to a large set of protein bands, but statistically significant differences were not calculated.

MALDI-TOF MS

We analyzed protein bands in the range of M_r 5 kDa to 16 kDa (Supplement Fig. 1). Between 5.4 kDa and 10.7 kDa, we identified non-specific lipid-transfer protein 2G (6.9 kDa), non-specific lipid-transfer protein 2P (7.0 kDa), ubiquitin (8.6 kDa) and non-specific lipid-transfer protein (9.5 kDa) as expected (data not shown).

Except in einkorn cultivars, we overall identified alpha-amylase inhibitor 0.28 at 12.2 kDa (band 4), and alpha-amylase/trypsin inhibitor CM3 at 15.6 kDa (band 7). We found evidence for several ATI at 13.3 kDa (band 5) with significant hits for alpha amylase/trypsin inhibitor CM2 in emmer cv. Osiris, for alpha amylase/trypsin inhibitor CM16 in emmer cv. Ramses, durum cv. Wintergold and durum flour, and for alpha-amylase inhibitor 0.19 in wheat cv. Akteur. At 14.7 kDa (band 6), the alpha amylase/trypsin inhibitor CM3 was also identified but with less high sequence coverage and signal intensity in all samples except in emmer cv. Osiris. In Tritordeum, also a significant hit for alpha-amylase/trypsin inhibitor CM16 was found for band 6. In general, hits with significant score correlated very well with calculated molecular weight without signal peptide for the protein identified. Complete results are shown in Supplementary Table 1.

We also matched measured m/z spectra with in silico digested ATI sequences of "reviewed" protein sequences in Uniprot database with omission of signal sequences which are usually cleaved during the maturation process.

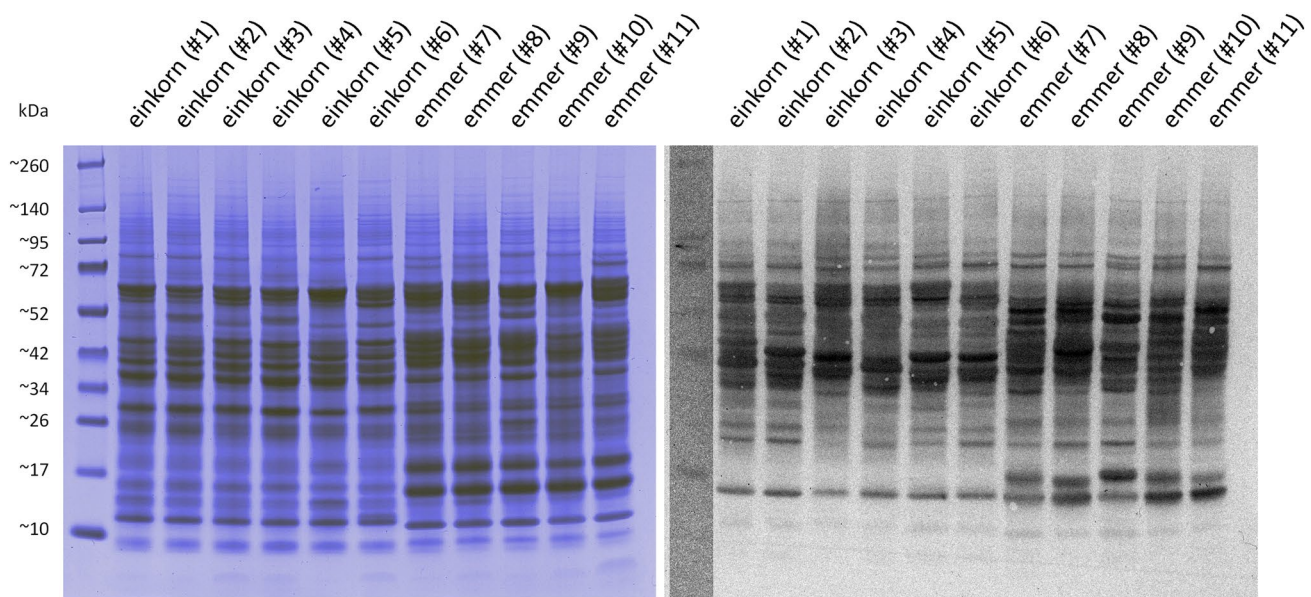


Fig. 3 Separation of albumins/globulins of six cultivars of einkorn and five cultivars of emmer in 4–12% gradient Bis–Tris polyacrylamide gels stained with Coomassie (left) and immunoblot with positive pool serum (right). Modified from [50]

In order to compensate possibly incomplete digestion, one missed cleavage site was allowed. The oxidation of methionine was considered as optional modification. Additionally applied ATI sequences for in silico analysis included Tri a 15.0101, Tri a 28.0101, Tri a 29.0101, Tri a 29.0201 and Tri a 40.0101 referenced by the WHO/IUIS, but not yet marked “reviewed” in Uniprot database. Sequences for Tri CMX from allergome.org were also considered.

We confirmed positive findings obtained in SwissProt database search as expected with higher sequence coverages for the respective sequences due to the omission of the signal peptide. With sequence coverages > 20%, evidence for ATIs in unidentified protein bands was found, e.g., Tri a 15 (UniProt P01083) in protein band 5 of emmer cultivars and durum samples, or Tri a 28 (UniProt P01084 and P01085) in protein bands 6 and/or 7 of spelt, Triticum and wheat. Sequence coverages of additional sequences agreed well or were identical with results of direct protein identification. In few cases a higher sequence coverage was observed, e.g., for Tri a 15.0101 protein band 6 in spelt cv. Franckenkorn. Considerable deviations in sequence coverages were found between sequences Tri a 40 (UniProt P16159) and Tri a 40.0101 (UniProt Q41540) in protein bands 5 and 6 of especially emmer cultivars and durum samples. Matched spectra of einkorn cv. Terzino and Tifi mostly showed low sequence coverages and intensities to the majority of ATI sequences. However, with 26.8%, the sequence coverage for trypsin/alpha amylase inhibitor CMX1/CMX3 in einkorn cv. Terzino at 13.3 kDa (band 5) was considerable, despite low intensity. Detailed results are shown in Supplementary Table 2.

In sum, in the excised protein bands, we could directly identify ATIs in emmer, durum, Triticum and wheat with significant probability between 12 kDa and 16 kDa, but not in the two einkorn cultivars.

Analyses of albumins and globulins from additional grains

Albumins and globulins of several varieties (einkorn, emmer, wheat, spelt, oat and one rye) and cultivars were submitted to electrophoretic separation on 4–12% gradient gels and immunoblotting in order to achieve a different resolution of proteins (Figs. 3 and 4).

The six cultivars of einkorn and five cultivars of emmer each showed high similarities in the protein pattern with some differences especially between $M_r \sim 42$ kDa up to ~ 62 kDa. In einkorn cultivars, strong protein bands at $M_r \sim 17$ kDa to ~ 15 kDa were absent as observed previously (Fig. 2). However, in the immunoblot we detected a clear positive signal in each cultivar of einkorn in the protein range of ATI at $M_r \sim 14$ kDa in contrast to findings in varieties Terzino and Tifi. A signal at $M_r \sim 17$ kDa was not detected. In emmer varieties, positive signals in the respective protein range were detected in accordance with previous results (Fig. 2). Of interest, among emmer cultivars #7 to #9, the immunoblot revealed a slight difference in size of the protein(s) of $M_r \sim 17$ kDa reactive to IgE which was not visible in the Coomassie stain.

We also compared wheat, spelt, and oat cultivars along with one rye cultivar (Fig. 4). Protein patterns were very similar between wheat and spelt with the exception of

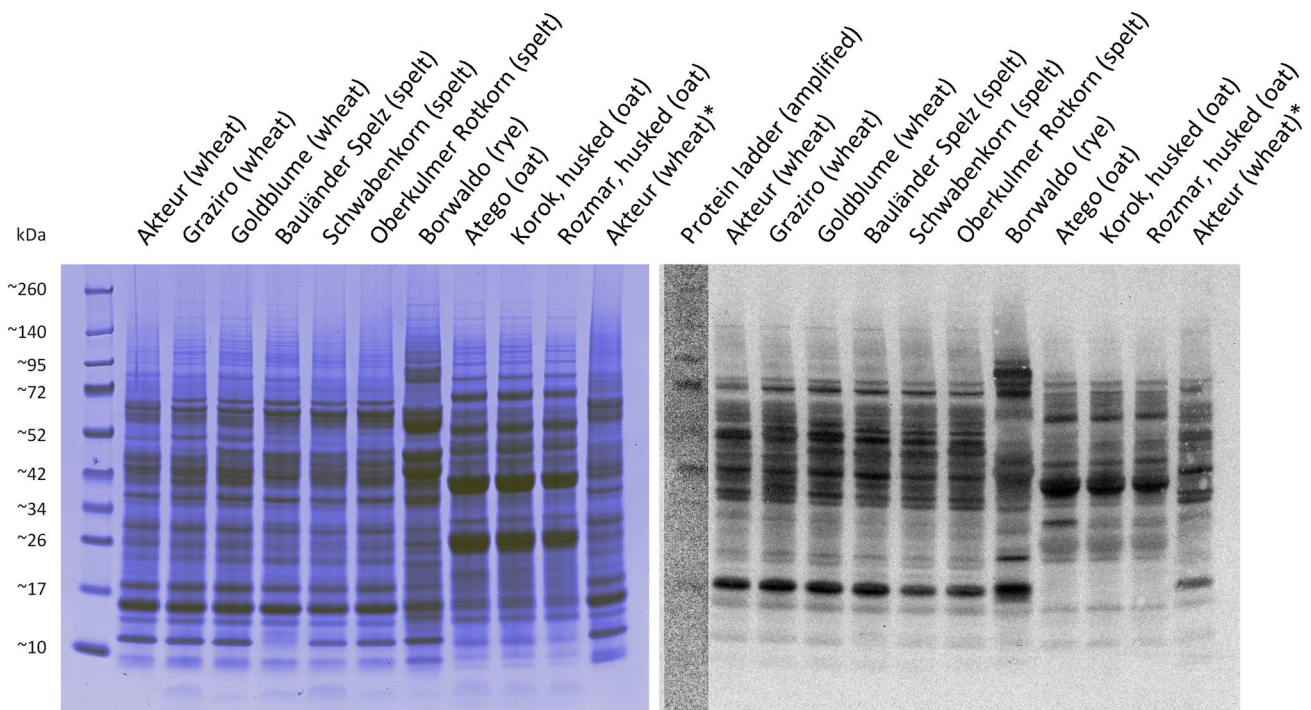


Fig. 4 Separation of albumins/globulins of wheat, spelt, oat and rye in 4–12% gradient Bis–Tris polyacrylamide gels stained with Coomassie (left) and immunoblot with positive pool serum (right). Modified from [50]

one band of $M_r \sim 12$ kDa missing in Bauländer spelt. We detected no significant differences between wheat and spelt in IgE-binding, also at the position of $M_r \sim 12$ kDa. The protein pattern of oats was very different from wheat and spelt. Binding of IgE was only detected between M_r 72 kDa and 26 kDa. Strong signals typical of ATIs at $M_r \sim 17$ kDa to ~ 11 kDa were not observed. In rye, strong signals were detected in the range of $M_r \sim 72$ to ~ 95 kDa, exceeding all cultivars used in this study. Again, a positive signal around $M_r \sim 17$ kDa for ATI was observed.

The negative controls showed low unspecific IgE-binding which was slightly higher in oat samples.

In sum, we identified no cultivar with a significantly reduced reactivity to IgE in pool serum of wheat-sensitized individuals in immunoblot analysis.

Discussion

We analyzed in detail the IgE-binding capacity to water/salt-soluble and water-insoluble protein fractions of selected diploid, tetraploid and hexaploid cultivars of the genus *Triticum*, and screened additional cultivars with respect to water/salt-soluble proteins only. Protein band patterns of the albumin/globulin fraction were very similar between tetraploid emmer and durum, and hexaploid spelt and wheat. Least similar were einkorn, oats and rye which correspond to

ploidy and phylogenetical relation. Significant dissimilarities occurred in the seed storage proteins (gliadins and glutenins) which have been subject to optimization in yield and baking characteristics for decades. Even among representatives of the same cultivar, protein patterns in the gliadin and glutenin fraction differed clearly, reflecting immense genetic diversity and plasticity for the respective genes. However, analysis of immunoblots with pooled serum from six individuals revealed no hypoallergenic cultivar of selected representatives of the genus *Triticum*. Our data, especially for the albumin/globulin fraction, give no evidence for the proposed breed-driven increase of allergenicity.

In contrast to other studies, e.g., the HEALTHGRAIN program [36], we did not determine the protein content by protein assays or the Kjeldahl method, but worked with equal volumes during the protein extraction and electrophoresis process. This approach circumvents the inaccuracy of protein assays which depend on the presence of basic amino residues (lysine, arginine) which are not equally abundant in proteins. Even if Coomassie staining may lead to underestimation of gliadins to glutenins due to different contents of lysine and arginine, these proteins were available in immunoblot for IgE to bind. The ratio of Coomassie and immunoblot ODs may be misleading for direct comparison of protein fractions, but provide information on IgE-recognition capacity of varieties and selected cultivars for each protein fraction. Our data show that albumins/globulins,

gliadins and glutenins in all varieties of the genus *Triticum* are highly allergenic. This result is not surprising because of the close phylogenetic relation which is associated with high sequence identities as shown for, e.g., non-specific-lipid protein among durum and wheat [37], or peroxidase in einkorn, durum and wheat [38]. In a quantitative IgE-binding dot blot analysis of water/salt-soluble extracts from 36 einkorn cultivars with each of the three wheat and durum cultivars, also no significant differences were found [39]. However, in the study of Klockenbring et al. especially IgA and IgE differed in binding affinities to proteins of the water/salt-soluble fraction of five cereals (one wheat, two spelt and two hybrid cultivars) in immunoblot and ELISA, reflecting cultivar-specific variations [31]. This result was explained with modified epitope structures (microheterogeneity) in related sequences of the respective protein. Another study was provided by Larré et al. [40], who compared one modern hexaploid (cv. Réctal) with one diploid ancient wheat (einkorn cv. Engrain) in ELISA and immunoblots. Based on 17 sera of a pediatric population and three adult individuals with ingestive allergy to wheat, the authors found higher levels of IgE to the modern cultivar in the majority of sera. In immunoblots of seven sera, similar results were obtained. Interestingly, two sera not positive in ELISA showed binding to proteins of Réctal, but not Engrain. No ATI-specific IgE-responses were found in the einkorn cultivar, and MS analysis was also negative. This result was interpreted as one possible explanation for the lower responses in ELISA to einkorn.

We analyzed protein bands in the range of M_r 5 to 15 kDa of the albumin/globulin fraction to investigate significant differences in both Coomassie staining and immunoblot. The range of 11 kDa to 15 kDa is typical of monomers of the ATI family when denaturing electrophoresis under reducing conditions is applied. In native form, they are classified as tetrameric, dimeric and monomeric with M_r 60, 24 and 12 kDa, respectively, according to structural characteristics and specificity to alpha-amylase. By MALDI-TOF MS, we detected several ATI (Tri a 15, Tri a 28, Tri a 29, Tri a 30.0101 and Tri a 40), except in cultivars of einkorn. As we used excised protein bands from a 1D SDS-PAGE, we applied strict conditions for bioinformatical identification and performed an in silico analysis of ATI peptides to the recorded spectra for confirmation. Our results match observations first reported by Bedetti et al. [41], who applied inhibition tests, and later confirmed by antibody-based assays and mass spectrometry by Zoccatelli et al. [42]. Most recently, the group of Köhler provided quantitative MS data on ATI content, showing that “ancient” spelt and emmer had even higher amounts compared to durum and bread wheat [43]. This finding might be of interest in the pathology of coeliac disease due to their ability to directly activate toll-like receptor 4 and inhibit digestive enzymes, causing impaired gluten

degradation [44–48]. ATI are also major allergens involved in IgE-mediated baker’s asthma and ingestive wheat allergy [49, 50]. Prandi et al. compared three cultivars of durum cultivated on three different areas and found significant differences in the content of alpha-amylase/trypsin inhibitor CM3 (Tri a 30) [51]. Baker et al. found a twofold range of ATI levels in 104 samples of Eastern soft wheat cultivars [52]. Similar results were found for another major allergen, ω 5-gliadin, of relevance for WDEIA patients with sensitization to this wheat component. Cultivars of einkorn [30] and spelt [25] with no or strongly reduced content of ω 5-gliadin were reported. However, there are considerable differences in the content of ATI or ω 5-gliadin depending on growing condition and cultivar. This could explain positive signals at $M_r \sim 14$ kDa in immunoblots of additional cultivars of einkorn which correspond to the molecular weight of monomers of ATI. As we did not perform MALDI-TOF MS of respective protein bands, we cannot exclude the presence or absence of ATI in these einkorn cultivars. However, mono-sensitization to one wheat component is rather rare in IgE-mediated wheat allergy [33, 49]. Thus, ancient grains are no hypoallergenic alternative in general since they still contain several IgE-reactive proteins of water/salt-soluble albumins/globulins as well as water-insoluble gliadins and glutenins. Altenbach et al. successfully reduced the content of ω 5-gliadin in modern bread wheat by gene silencing, but still observed IgE-reactivity to many other proteins [53]. Nonetheless, the authors suggest transgenic wheat as a means to reduce sensitization to the respective component in naïve individuals which would also limit cross-reactions occurring between similar epitopes of, e.g., ω 5-gliadin and other gliadins. Growing conditions and cultivar may also explain contradictory results of De Santis et al. who compared old and modern durum genotypes and found higher levels of ω 5-gliadin in old genotypes [54], whereas Ribeiro et al. reported generally higher amounts of ω 5-gliadin in modern wheat than in spelt or durum [25].

Our results on water-insoluble gliadins are also in accordance with studies on coeliac disease which showed that ancient wheat lines can even exceed modern wheat in toxicity, independent of ploidy level and cultivation (organic or conventional) [25, 28], although genotype and environmental factors influence expression levels of especially seed storage proteins [54–58]. As repeatedly shown, there is a high natural variation within cultivars [25, 59, 60].

In contrast to clinical test systems, wheat is usually consumed in a highly processed form and not as raw flour. Processing of cereals has repeatedly been a matter of debate [61, 62]. For individuals suffering from coeliac disease, transglutaminase-mediated transamidation was shown to be a promising technology to produce bread with significantly reduced levels of gluten [63, 64]. Enzymatic degradation has also been suggested [65]. Clinical studies and the

application to individuals with IgE-mediated wheat allergy are still pending. On the molecular level, fermentation, enzymatic treatment and especially thermal heating alter proteins by glycation of residues (Maillard reaction) and thus their allergenic potential. It could be argued that sensitization is likely to happen towards these newly formed glycosylamines or denatured proteins which should be considered in diagnostic tests as one means to maintain specificity and clinical relevance. Some studies investigated the influence of thermal treatment on IgE-binding, e.g., Klockenbring et al. compared the albumin/globulin fractions of five cereals (one wheat, two spelt and three hybrids) in native and (heat treated) denatured form in ELISA. The authors found a significantly reduced IgE-binding to denatured proteins except in the wheat (cv. Astron) using pooled sera of six individuals without detailed clinical phenotyping. De Gregorio et al. [66] showed by ELISA and immunoblot that water and salt-soluble proteins from heat-treated wheat foods (bread and cooked pasta) have a strongly reduced IgE-binding capacity compared to raw flour in 13 adult patients and one 2-year-old. However, these results would need confirmation in a larger setting and also in a pediatric population. Nonetheless, processed wheat foods still have allergenic potential, and binding of IgE to unprocessed raw flour indicates contact to not only processed flour. However, for the clinician, specific IgE indicates sensitization, but not necessarily clinical reaction towards wheat ingestion. Furthermore, cross-reaction with grass is quite frequent and an obstacle in the diagnostic workup of wheat allergy [67–69]. The pool serum used in this study was strongly positive in the sxl panel which detects specific IgE to house dust mite, cat and dog dander, timothy grass, common silver birch, cultivated rye, mugwort and mold fungus. Thus, unspecific, clinically irrelevant cross-reaction of inhalation-related IgE to wheat is very likely. On the other hand, IgE is the prerequisite for the development of allergic symptoms which may also occur due to cross-reactions even with mild symptoms. The use of pooled serum also bypasses the risk of biased results due to the limited epitope-reactive IgE-panel in individual patients. Another possible issue of the assessment of sensitization is the origin of the test material and mismatches with the individual or population investigated. Comparing self-coupled and commercially available “European” antigens on ImmunoCAP (Phadia, Uppsala, Sweden, now Thermo Fisher), correlation coefficients of 0.92 for wheat and 0.88 for spelt were reported for an adult study population in Australia [32]. Klockenberg et al. hypothesized that previous exposure to wheat foods may explain positive “experiences” with spelt (no or less allergic reactions) because sensitization was initiated by wheat proteins that slightly differ from spelt in crucial epitopes for IgE present. Systematic studies on the fate of wheat allergens during processing and the sensitization history would be helpful to evaluate positive reactions of IgE

to raw flour in recent test systems as concerns clinical implications. As ELISA and immunoblots can neither resolve complex protein mixtures nor the heterogeneity of epitopes on one protein, component-resolved diagnostics and epitope mapping are the methods of choice needed for a comprehensive assessment of selection strategies. These should focus on immune-dominant epitopes for further breeding because as exemplified for water-insoluble proteins, total gluten or gliadin/glutenin determination is not sufficient to resolve allergenicity [25].

From a phylogenetical point of view, as shown by Pfeifer et al. all subgenomes in modern wheat contribute on the transcriptional level to spatiotemporal processes during endosperm development [70]. Despite the lack of subgenomic dominance in modern bread wheat, gene families located on the A genome were found to show a bias related to seed formation [35]. Provided precise phenotyping and broad screening, modern and ancient *Triticum* varieties with alleles suitable for genome editing could be identified in order to create cultivars with at least reduced levels of major allergens. Despite the fact that ancient cereals are hardly less allergenic, they enrich the daily diet of tolerant individuals. As pointed out by Longin and Würschum in their holistic approach, ancient varieties should be considered for the sake of diversification and satisfaction of consumers’ needs, but they are hardly sufficient to feed the growing population due to low yield and traits that are incompatible (height, hulled grains) to recent agricultural practices [71]. In the case of einkorn, its poor baking quality restricts its use to the preparation of cookies and pasta than bread-making. In the context of climate change, rapid selection and improvement of cultivars has become an important issue which underlines the necessity of phenotyping, genetic screening and comprehensive proteomic databases [72–75]. Despite limitations discussed, assessment of allergic potential of molecular bred cultivars could be considered *in silico* beforehand [76]. Further insight into the genetic basis of nutritional and constitutional traits is needed to retain and improve adaptations to (a)biotic factors in modern, wild and new cereals [73].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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