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Krisztina Takács^a; Jenő Szamos^a; Tamás Janáky^b; Marianne Polgár^c; Éva Gelencsér^a

^a Department of Food Safety, Central Food Research Institute, Unit of Biology, Budapest, Hungary ^b

Department of Medical Chemistry, University of Szeged, Dóm tér, Hungary ^c Center of

Gastroenterology, Madarász Children's Hospital, Hungary

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Immune-analytical detection of the cross-reactive major cereal allergens

Krisztina Takács^{a*}, Jenő Szamos^a, Tamás Janáky^b, Marianne Polgár^c and
Éva Gelencsér^a

^aDepartment of Food Safety, Central Food Research Institute, Unit of Biology, H-1022 Budapest, Herman Ottó út 15, Budapest, Hungary; ^bDepartment of Medical Chemistry, University of Szeged, H-6720 Szeged, Dóm tér 8, Hungary; ^cCenter of Gastroenterology, Madarász Children's Hospital, H-1131 Madarász Viktor u. 22/24, Hungary

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Investigation of allergens of celiac disease and cereal allergy has become important for scientific research. Immune-blotting is currently one of the most appropriate methods applied for the identification of potential allergens. The research was focused on the examination of in vitro allergenic activity of proteins in domestic wheat, triticale, rye, barley, rice, corn, buckwheat and amaranth cultivars. The IgA/IgE reactive epitopes of water/salt- and alcohol-soluble protein fractions, respectively, were detected with IgA antibodies of celiac and IgE antibodies of wheat allergic human sera. Further efforts were made to separate the alpha-amylase inhibitors from the water/salt-soluble protein fraction of wheat by DEAE-cellulose anion-exchange chromatography and to confirm their presence on native gel. The most frequently recognised IgE reactive proteins (27) by wheat allergic human sera were identified by liquid chromatography coupled with mass spectroscopy and accession to databank supply. The pepsin resistance of identified wheat allergens was assessed directly on PVDF membrane-blotted protein bands.

Keywords: alpha-amylase inhibitor; allergen; immune-blotting; LC-MS/MS; database

Introduction

Cereals have had a significant role in human nutrition for a long time. Beside their energy content (carbohydrate) they are essential sources of further nutrients such as vitamins, minerals, dietary fibres and proteins. Among cereals, wheat is one of the most important crops, and is grown in the widest area. Despite its favourable nutritional benefits, wheat is one of the most prevalent allergen sources for patients suffering from celiac disease and/or wheat allergy. In the background of the two different sorts of diseases, there are different immunological processes, in which different allergens play a role in their elicitation. According to our present knowledge the strong allergen components, most often causing wheat allergy are the alcohol-soluble gliadins (α -gliadins 40 kDa, ω 5-gliadins 65 kDa), the water-soluble alpha-amylase inhibitors (AAIs; 0.53 15 kDa, CM3 14 kDa, tetramer CM3 60 kDa) and the non-specific lipid transfer proteins (Lehto et al., 2003; Palosuo et al., 1999; Pastorello et al., 2007; <http://www.informall.eu.com>). These components can cause a

*Corresponding author. Email: k.takacs@cfri.hu

wide range of symptoms. The sensitisation can occur either via the respiratory organs or the gastrointestinal tract. In the case of celiac disease, the toxic allergenic factors are the gliadins and the prolamins have a similar immunological structure to that of gliadins (e.g. rye secalin, barley hordein, oat avenin), which can cause serious atrophy of the intestinal villi of sensitised patients. The only therapy for both diseases is the avoidance of the symptom-causing allergens and intolerance factors, namely the consumption of allergen-free foodstuffs. Since numerous wheat proteins with allergenic properties have been revealed, the research on their chemical-, immunological-, clinical- and molecular-based examinations have become important. These examinations are very difficult because of the heterogeneity of the clinical symptoms and the wheat allergens. The potential protein allergens can be characterised by immunological methods (ELISA, 1D and 2D immune-blotting) based on their protein structure, then produced and characterised biotechnologically as recombinant protein, as well as announced in databases. The recombinant technology facilitated the production of different types of allergens in high yield and purity with IgE- and IgA-binding capacity compared to the natural allergens.

The pathomechanism of celiac disease and wheat allergy are more or less well known, but only scarce information is available on the target antigens for both diseases. In the case of celiac disease gluten is the major allergen, and the predominance of gluten-specific IgA and IgG antibodies characterises the reaction. The levels of IgE antibodies against the reactive wheat allergen proteins are increased in cases of wheat allergy.

IgA/IgE-reactive antigens in wheat were compared by Constantin, Huber, Granditsch, Weghofer, and Valenta (2005) using sera from patients with celiac disease and food allergy to wheat by one-dimensional (1D) immune-blotting. IgA antibodies from celiac patients and IgE antibodies from allergic patients recognised distinct profiles of wheat antigens. However, some proteins were recognised by both sera, and big differences in their immune-blotting profiles were still characteristic. Celiac patients showed a rather uniform IgA reactivity to wheat antigens, whereas wheat allergic patients exhibited a rather heterogeneous IgE reactive profile. It was demonstrated that wheat contains antigens/epitopes which are preferentially recognised by celiac patients, whereas others elicit IgE-mediated food allergy. It could be concluded that the nature of a food antigen might influence the quality of the pathological immune response in the gut, and lead obviously to different diagnosis and therapy in both diseases.

Our efforts were to investigate other cereals (rye, barley, triticale, oat, corn, rice) and pseudo-cereals (amaranth, buckwheat) than wheat. The novelty of our research study is that celiac and wheat allergic sera equally were used for all the samples involved. Furthermore, in the case of wheat an initial chromatographic step based on DEAE-cellulose was elaborated to separate AAIs as the most allergenic proteins according to literature data.

Materials and methods

Materials

Domestic cereal (wheat, rye, barley, triticale, oat, corn, rice) and pseudo-cereal (amaranth, buckwheat) cultivars were provided by the Cereal Research Non-profit

Company (Szeged, Hungary) and Klorofill Bt. (Kecskemét, Hungary). Five cultivars were tested per sort, except amaranth, of which only three cultivars were examined. These were bread wheat (*Triticum aestivum*) GK Tisza 2004; speltin wheat (*Triticum spelta*) ÖKO-10 2004; durum wheat (*Triticum durum*) D-78 2004; bread wheat (*Triticum aestivum*) GK Cipó 2004; bread wheat (*Triticum aestivum*) CY-45 Grandstar 2001; barley (*Hordeum vulgare*) spring STC I. 22-03 2004; barley (*Hordeum vulgare*) autumn GK Rezi 2004; barley (*Hordeum vulgare*) autumn GK Rezi 2005; nude barley (*Hordeum nuda*) autumn C3/05 2005; nude barley (*Hordeum nuda*) autumn GK Árpád 2005; rye (*Secale cereale*) Amilo 2004; rye (*Secale cereale*) Rapid 2004; rye (*Secale cereale*) autumn GK Wibro 2004; rye (*Secale cereale*) Matador 2004; rye (*Secale cereale*) Gamet 2004; triticale (*Triticosecale* sp.) spring Kargo 2004; triticale (*Triticosecale* sp.) spring EMBR 18 2004; triticale (*Triticosecale* sp.) spring GK korai 2004; triticale (*Triticosecale* sp.) spring Wanad 2004; triticale (*Triticosecale* sp.) spring GK Idus 2004; nude oat (*Avena nuda*) spring GK Zalán 2004; oat (*Avena sativa*) spring GK Iringó 2004; oat (*Avena sativa*) autumn GK Impala 2004; oat (*Avena sativa*) spring GK Kormorán 2005; oat (*Avena sativa*) spring GK Pillangó 2005; corn (*Zea mays*) Sze TC 269 2004; corn (*Zea mays*) Ella SC 2004; corn (*Zea mays*) Sze SC 352 2004; corn (*Zea mays*) Bella TC 2004; corn (*Zea mays*) Veronica SC 2004; rice (*Oryza sativa*) Janka 2003; rice (*Oryza sativa*) Bioryza 2003; rice (*Oryza sativa*) Risabell 2003; rice (*Oryza sativa*) Ábel 2003; rice (*Oryza sativa*) Oryzella 2003; amaranth (*Amaranthus moleros*) GK Róza 2003; amaranth (*Amaranthus cruentus*) GK Maros 2005; amaranth (*Amaranthus hypochondriacus*) Edit 2004; buckwheat (*Fagopyrum esculentum*) Hajnalka 2004; buckwheat (*Fagopyrum esculentum*) 430 tribe 2003; buckwheat (*Fagopyrum esculentum*) P425 tribe 2004; buckwheat (*Fagopyrum esculentum*) P427 tribe 2004; buckwheat (*Fagopyrum esculentum*) P429 tribe 2004.

Individual anonym sera from celiac and wheat allergic humans were originated from the cohort studies and clinically certified by Dr Polgár (Madarász Children's Hospital, Budapest, Hungary). TrimW as wheat AAI was bought from Nutricepts (USA).

Preparation of samples

Cereal and pseudo-cereal seeds were milled by a Retsch GRINDOMIX GM200 grinder (15 sec, 5000 rpm, following 60 sec, 8000 rpm) yielding 80% flour which was sieved to flour size (250 µm). After milling, the knife and the tank of the grinder was washed with water and detergent, and then dried, and the sieve was also cleaned with a hoover and detergent to avoid the cross-contamination.

Purification of soluble fractions

Water/salt-soluble fraction

One gram of the milled and sieved flour was extracted with 4 ml of 50 mM Tris-HCl buffer (pH 8.5) at 4 °C by shaking for an hour. The suspensions were centrifuged (10 min., 13,000 rpm), then an appropriate dilution of supernatants was applied for further analyses.

Alcohol-soluble fraction

One hundred milligrams of flour was extracted with 1 ml of 70% ethanol at room temperature by shaking for half an hour. The suspensions were centrifuged ($500 \times g$, 10 min), then appropriate dilution of supernatants applied for further analyses.

Alpha-amylase inhibitor (AAI) rich fractions of wheat

In case of wheat samples, DEAE-cellulose anion-exchange chromatographic method was developed and applied to obtain protein fractions enriched in AAI from the water/salt-soluble fractions. Two fractions were separated with fixed condition (2 cm long column filled with 4 ml DEAE-cellulose, 2.75–2.9 ml loaded sample, 50 mM Tris-HCl start buffer (pH 8.5), 0.15 M NaCl contained 50 mM Tris-HCl eluting buffer (pH 8.5)). The absorption of fractions was measured by spectrophotometer (280 nm). All the fractions were concentrated with UF-membrane (Millipore Centricon Plus-80 Centrifugal Filter Devices with Ultracel PL membrane, 10,000 Da cut-off).

Alpha-amylase inhibitor activity

The AAI activity was assayed spectrophotometrically by Murao, Goto, Matsui, Ohyama, and Arai (1981). The presence of the AAI activity was confirmed on 10% native polyacrylamide gel according to the method of Henson and Stone (1988) with small modifications. Following the protein separation, the gels were immersed in 0.0025% alpha-amylase (Sigma; 1.37 U) containing extraction solution (40 mM Tris, 1 mM CaCl_2 , pH 8.0) for 20 min, then in 1% starch containing extraction solution at room temperature for 20 min. The gels were rinsed with distilled water, and then stained with potassium iodide/iodide solution (1 mM KI, 0.5 M I_2). The stained gels were rinsed with distilled water. Blue spots on pale yellow-coloured gels showed the presence of AAI in the samples.

Development of antiserum to the AAI enriched fraction of wheat TrimW

Antibodies to wheat AAI (rabbit anti-TrimW2nd antibody) were developed in rabbits according to the method of Harboe and Ingild (1973).

SDS-PAGE and immune-blotting

Combination of SDS-PAGE with immune-blotting was used for the determination of allergenic proteins. SDS-PAGE was performed according to Laemmli (1970) following the instruction manual of Mini-PROTEAN 3Cell (BioRad) using 6%/15% stacking/resolving gel. Separation was carried out at 200 V for approximately 65 min at room temperature. After electrophoresis, the separated proteins were fixed on gel with 20% TCA for 20 min, gel was then soaked in acetic acid/ethanol washing liquid (3×10 min). The staining was performed with Coomassie Brilliant Blue R-250 (Reanal, Hungary). The background staining was removed by 10% acetic acid. In case of immune-blotting, the separated proteins were subsequently transferred to PVDF membrane (Millipore, 0.45 μm) by semi-dry blotting (BIO-RAD Trans Blot

SD Semi-dry Transfer Cell) for 60 min at 0.25 V, 0.08 mA/cm². The blotting procedure was performed according to the instructions of the Millipore membrane brochure. The immune-reactive proteins were identified with anti-gliadin antibody raised in rabbit (SIGMA), with anti-TrimW2nd antibody produced in rabbit (CFRI, Hungary), or with individual human sera from patients suffering from wheat allergy with increased IgE antibodies to the toxic wheat protein allergens or celiac disease with increased IgA antibodies to gliadin. The binding patterns were visualised using a substrate solution containing 4-chloronaphtol (Sigma), H₂O₂ and ethanol in 16 mM phosphate buffered saline solution. Image analyses of gels and blots were carried out with BIO-RAD Gel Doc 2000 system.

Identification of proteins as allergens by liquid chromatography coupled with mass spectroscopy (LC-MS/MS) and Uniprot trembl database

To identify allergenic proteins from stained gel protein bands, liquid chromatography coupled with mass spectroscopy (LC-MS/MS) and Uniprot trembl database were used. The identification was performed at the University of Szeged, Faculty of General Medicine, Department of Medical Chemistry.

Stained gel was washed for three days by soaking in distilled water, and then the desired protein bands were cut out with lance and transferred into 1% acetic acid containing Eppendorf tubes and kept at 4 °C until the further preparations.

The gel particles were washed three times with 0.1 M ammonium-hydrogen-carbonate buffer (pH 8.0) for 20 min, then with acetonitrile for 20 min until discolouration. The particles were treated by 10 mM DL-dithiothreitol (DTT) for 30 min at 56 °C, and then washed again with 0.1 M ammonium-hydrogen-carbonate buffer (pH 8.0) for 10 min, and finally with acetonitrile for 10 min. Furthermore, the gel particles were treated with 50 mM iodoacetamide (alkylating agent) for 45 min at room temperature in dark place followed by a thorough process of washing for 10 min with 1:1 ratio of 0.1 M ammonium-hydrogen-carbonate buffer (pH 8.0) and bidistilled water, then simply with acetonitrile for 10 min. The samples were then freeze-dried. The freeze-dried samples were saturated with trypsin solution for 30 min on ice, and then digested for 16 hours at 37 °C with addition of mixture of 0.1 M ammonium-hydrogen-carbonate buffer (pH 8.0) and bidistilled water.

The reaction was stopped with 70 µl of 5% formic acid. The digested products (peptides) were re-extracted three times with a 50 µl mixture of 50% acetonitrile and 5% formic acid for 20 min, and additionally centrifuged by SpeedVac vacuum-centrifuge to be separated from the solvent. The samples were re-dissolved in 0.1% formic acid, and then were analysed by LC-MS/MS method with AGILENT 1100 equipment besides fixed parameters (LC-columns: Zorbax 300SB-C18, 5 µm, 5 × 0.3 mm; Zorbax 300SB-C18, 3.5 µm, 150 × 0.075 mm; loaded amount of samples to the column: 5 µl). MS/MS data were evaluated by Uniprot trembl database search with SpektrumMill inhouse server.

In vitro digestion by pepsin on PVDF membrane

The determination of the resistance of allergenic proteins towards pepsin was carried out on PVDF membrane-blotted proteins. The digestion was performed with

shaking in 0.32% pepsin in 30 mM NaCl (pH 2.0; FAO/WHO, 2001) for 0, 30 min and/or 60 min at 37 °C. Then the pepsin solution was removed from the membrane by washing three times with PBS buffer (pH 7.4) for 10 min. After that the membrane was shaken in 1% BSA blocking solution for 1 hour. The immune-blotting procedure was carried out by following the PVDF membrane brochure instructions (Millipore).

Results and discussion

The main objective was to identify allergenic proteins in Hungarian wheat cultivars and immunologically cross-reactive proteins in other cereals and pseudo-cereals by means of SDS-PAGE and immune-blotting using celiac and wheat allergic human sera for their recognition. In order to conduct a comparative study, wheat and samples with celiac toxicity such as triticale, rye, barley and oat as well as non-toxic ones such as rice, corn, amaranth and buckwheat were included.

The two major groups of wheat allergens are the gliadins and AAIs therefore the pattern of proteins were assessed by anti-gliadin rabbit IgG (Sigma) and anti-TrimW2nd rabbit IgG antibodies (CFRI).

The AAI specific polyclonal antibodies were developed against AAI rich protein antigen. For this purpose a commercially available AAI preparation of wheat origin (TrimW, Nutricepts) was further purified for AAIs with a home-developed DEAE-cellulose anion-exchange chromatographic procedure which was confirmed by measuring the AAI activities.

This concentration step increased the initial AAI activity (10%) in the first non-column bound fraction up to 67% and in the second column bound fraction up to 95%. Since the bound fraction eluted from the DEAE-column contained higher enzyme activity and its purity also corresponded to the immunising requirements, this fraction was used as an antigen.

The recognition patterns of anti-gliadin rabbit IgG and the anti-alpha-amylase rabbit IgG antibodies showed high similarity, namely, the recognised proteins had a similar molecular size pattern (the immune-blotting data are not shown).

The common immune-structure of the antigens can be due to the fact that the majority of the gliadins (except $\omega 5$ of gliadin) according to their function, belong to the alpha-amylase/trypsin inhibitor superfamily (<http://www.informall.eu.com>). These results showed that the target proteins in samples could not be easily identified. Therefore, the AAI and gliadin antigens obtained from the stepwise extraction of the soluble proteins, the water/salt-soluble and the alcohol-soluble fractions, were run separately by SDS-PAGE combined with immune-blotting. Each cereal/pseudo-cereal species is represented with one sample per species in the following figures concerning the SDS-PAGE separation and immune blots as significant differences between the certain species were not observed.

For ethanol-soluble proteins Figure 1a shows that wheat gliadins located between the range of 37–54 kDa are in conformity with the literature (Skerritt, Andrews, Blundell, Beasley, & Bekes, 1994). Amaranth, buckwheat and rice did not contain extractable prolamins. The pattern of ethanol-soluble proteins of the wheat-cross-reactive crops and the corn were different. The molecular size of the majority of prolamins for rye, triticale and barley were similar (37–54 kDa), while for oat (29–37 kDa) and corn (26–29 kDa) the bands were different. Contrary to

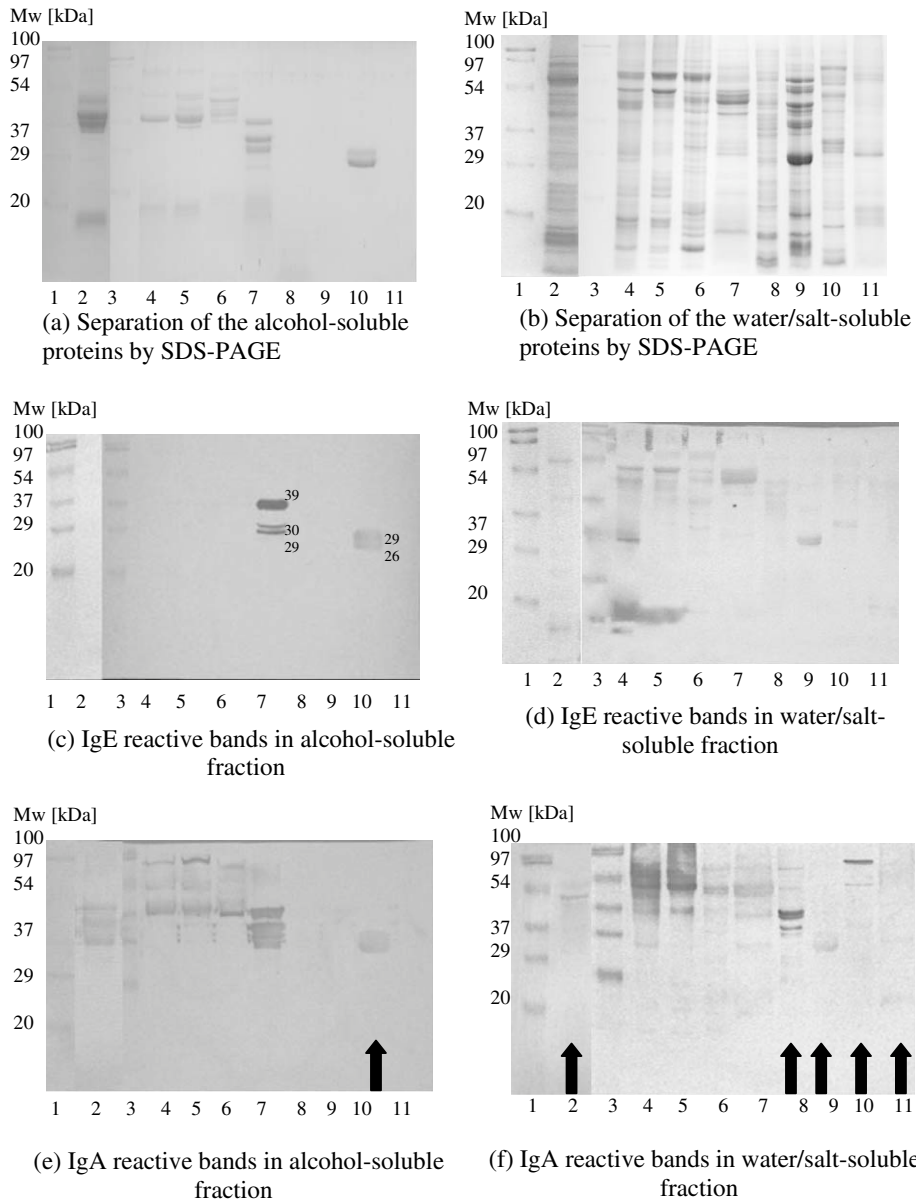


Figure 1. SDS-PAGE and immune-blotting photos of cereals and pseudo-cereals. 1. Protein marker (Mw); 2. Wheat gliadin (Sigma); 3. Protein marker (Mw); 4. Rye (*Secale cereale*), Amilo 2004; 5. Triticale (*Triticosecale* sp.), spring, EMBR 18, 2004; 6. Barley (*Hordeum vulgare*), spring, STC I. 22-03, 2004; 7. Oat (*Avena nuda*), spring, GK Zalán, 2004; 8. Amaranth (*Amaranthus cruentus*), GK Maros, 2005; 9. Buckwheat (*Fagopyrum esculentum*), 430 tribe, 2003; 10. Corn (*Zea mays*), Ella SC, 2004; 11. Rice (*Oryza sativa*), Bioryza, 2003.

the uniformity of the alcohol-soluble proteins, the water/salt-soluble fractions of cereals/pseudo-cereals gave more bands at different molecular weights as shown in Figure 1b.

For immune-blotting wheat allergic (IgE) and celiac (IgA) human sera were used for monitoring the immune-reactivity of proteins in both soluble fractions of all the cultivated samples. The immune-reactive bands in wheat and proteins having cross-reactivity with these specific sera were determined.

In case of the wheat (Figure 1c, lane 2) it was observed that the alcohol-soluble gliadins of the wheat cultivars did not give any IgE-immune-reactivity against wheat sensitive sera. The IgE reactive bands recognised by wheat sensitive sera in water/salt-soluble fraction (Figure 1d, lane 2) and IgA reactive bands recognised by celiac patient's sera in alcohol-soluble fraction (Figure 1e, lane 2) were different in their profiles but some bands were recognised by both antibodies. The results are comparable with the results obtained by Constantin et al. (2005) for the whole protein extract. Surprisingly the celiac patient's sera recognised some IgA reactive bands in the water/salt-soluble protein fraction as well (Figure 1f, lane 2). Our previous experience has shown that the wheat allergic patient's sera had a specific IgA recognition pattern as well (data not shown). Therefore, it was supposed that these patients could have additional wheat allergy problems as well. The reactivity profile of the celiac patients was considerably more uniform than that of the allergy-diseased patients. The sera from allergy-diseased patients showed remarkably heterogeneous IgE-reactivity depending on the individuals.

The IgE reactivity of the cross-reactive (Figure 1c, lanes 4, 5, 6, 7) and non-cross-reactive (Figure 1c, lanes 8, 9, 10, 11) samples in the prolamin fractions did not give large-scale variability. In each case, strong cross-reactivity with avenins of oat (29–39 kDa) and a weak reaction with zeins of corn (26–29 kDa) were observed. Weichel et al. (2006a) has observed similar cross-reactivity with the corn. In case of rye, triticale and barley, only certain sera gave weak IgE reactive protein bands at 39 kDa.

The IgE reactive bands were different when reacted with individual wheat allergic sera in the water/salt-soluble fraction and were located at the range of 10–100 kDa (Figure 1d).

The IgA antibodies of the individual celiac sera reacted with almost all the proteins in the range of 37–54 kDa of the prolamin fraction (Figure 1e) giving uniform pattern with some characteristically strong and weak bands and also with a lot of proteins in the water/salt-soluble fraction between the range 16–97 kDa (Figure 1f). It was an unexpected and surprising result that the corn prolamines (zeins), and some water/salt-soluble proteins derived from corn, amaranth, and buckwheat show IgA reactive bands. According to our present knowledge (CX/NFSDU ALINORM 08/31/26, Appendix III) these proteins do not cause clinical symptoms (alterations in the villi of the human intestinal tract) in celiac patients. It was supposed that the *in vitro* recognition not necessarily related to the pathomechanism of celiac disease but the patients could have additional wheat allergy disease problems. Therefore, further examination of these reactive proteins must be carried out.

In our further work wheat, AAIs were studied in respect of IgE reactivity. The water/salt-soluble fractions were represented with two AAI-enriched fractions, which were produced by a home-developed DEAE-cellulose anion-exchange chromatographic separation. The purpose of concentration of AAIs was to facilitate their recognition by wheat allergic sera in the water/salt-soluble fraction.

The albumin/globulin extraction with 50 mM Tris-HCl buffer (pH 8.5) introduced by Weiss, Vogelmeier, and Görg (1993) as a modification of the extraction with 0.15 M NaCl (Osborne, 1907), made it possible to perform DEAE-cellulose chromatography. Weiss et al. (1993) separated wheat water/salt-soluble proteins between pH 5.0 and pH 9.0 isoelectric points (pIs) by means of 2D gel electrophoresis. On immune-blotting the pIs of IgE reactive AAI proteins recognised by wheat sensitive human sera were found in the pH range of 4.0–8.5. The pH of the start buffer for the chromatography in our study was adjusted to 8.5. As we used the extraction buffer (50 mM Tris-HCl buffer (pH 8.5)) of the samples as a start buffer for the chromatography, the compatibility of the electrophoretical and chromatographical separation was also well feasible. Moreover, our aim was to assure the reproducibility of extraction, which was achieved with the appropriate homogeneity of samples (flour granule size: 250 µm), ratio of volume, precisely kept temperature and time. In case of all samples two fractions were collected by column chromatography. The first fraction contained the unbound proteins, which were eluted with 50mM Tris-HCl buffer (pH 8.5), while the bound proteins could be eluted in the second fraction by 15% NaCl containing 50 mM Tris-HCl buffer (pH 8.5).

The most frequently recognised IgE reactive proteins in the concentrated fractions (first and second fractions) of AAIs of domestically cultivated wheat varieties and also in water/salt-soluble fraction of other cereals and pseudo-cereals, giving the strongest bands towards the majority of wheat allergic human sera were analysed by LC-MS/MS after SDS-PAGE separation, and identified from the Uniprot trembl database.

Figure 2 shows the place of the protein bands cut out from gels and sent for the peptide-based identification (K1-29). Tables 1, 2 and 3 summarise the data of the identified proteins. MS–MS data was evaluated by SpectrumMill inhouse server using Uniprot trembl database to find the proteins of the target species. The 1.6 Da and 0.6 Da accuracy was allowed to the precursor ion and fragments, respectively. Among the results, those proteins are listed, which could be identified with a minimum of two peptides and the unique score of those was at least 20. A list of the homolog proteins can be found in the (Additional accession numbers) column.

It's important to emphasise the results of Table 1. Among potential allergenic wheat proteins from the database with appropriate peptide covering rate xylanase inhibitor, AAI (monomer, dimer, CM2, CM3, CM16, 0.19), serpin, tritin, ns-LTP, β-amylase, superoxide dismutase and avenin-like wheat protein were especially found. It was proven that AAIs, which are reported in the literature as the most important allergens and serpins, which are found in the most recent research as new allergens (Akagawa et al., 2007; Weichel et al., 2006a), and HMW glutenin (Weichel, Glaser, Ballmer-Weber, Schmid-Grendelmeier, & Cramer, 2006b; Weichel et al., 2006a) could be detected and identified as allergens in domestically cultivated wheat varieties and commercially available TrimW product. Comparing proteins identified by other research with these results it can be said that the differences could be due to the different wheat cultivars, the heterogeneity of the sick population, and the different methods used for the protein identification.

On the allergens identified, it can be expected that there is a wide scale of the allergen-source, which can cause symptoms. As symptoms can change with wheat

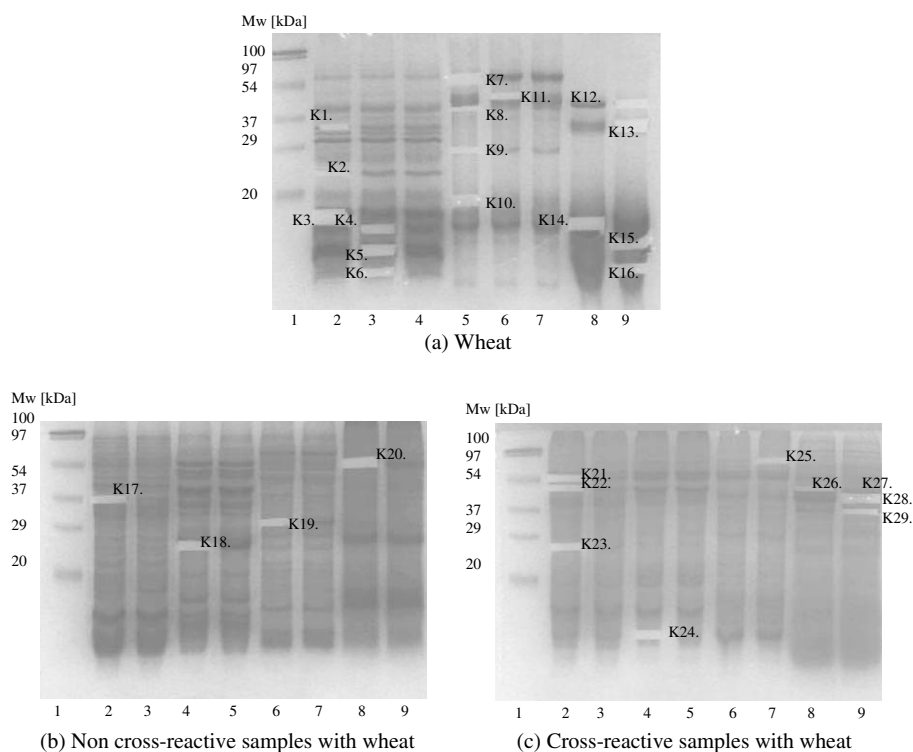


Figure 2. Most frequently recognised IgE reactive bands by wheat sensitive human sera in water/salt-soluble fractions of cereals and pseudo-cereals submitted to database identification. (a) 1. Protein marker (Mw); 2–4. *T. aestivum*, GK Cipó, 2004 – 1st fraction (K1 34 kDa, K2 24 kDa, K3 16 kDa, K4 15 kDa, K5 13 kDa, K6 10 kDa); 5–7. *T. aestivum*, GK Cipó, 2004 – 2nd fraction (K7 67 kDa, K11 50 kDa, K8 45 kDa, K9 29 kDa, K10 19 kDa); 8–9. TrimW-2nd fraction (K12 45 kDa, K13 37 kDa, K14 16 kDa, K15 14 kDa, K16 12 kDa). (b) 1. Protein marker (Mw); 2–3. Amaranth (*Amaranthus cruentus*), GK Maros, 2005 (K17 36 kDa); 4–5. Buckwheat (*Fagopyrum esculentum*), 430 tribe, 2003 (K18 25 kDa); 6–7. Corn (*Zea mays*), Ella SC, 2004 (K19 29 kDa); 8–9. Rice (*Oryza sativa*), Bioryza, 2003 (K20 52 kDa). (c) 1. Protein marker (Mw); 2–3. Rye (*Secale cereale*), Amilo, 2004 (K21 64 kDa, K22 48 kDa, K23 26 kDa); 4–5. Triticale (*Triticosecale* sp.), spring, EMBR 18, 2004 (K24 12 kDa); 6–7. Barley (*Hordeum vulgare*), spring, STC I. 22-03, 2004 (K25 79 kDa); 8–9. Oat (*Avena nuda*), spring, GK Zalán, 2004 (K26 49 kDa, K27 46 kDa, K28 43 kDa, K29 38 kDa).

variety, so diagnosis becomes more difficult. That is the reason why an extract from the mixed wheat cultivars should be applied for screening the patients suffering from wheat allergy.

On native polyacrylamide gel the presence of AAIs of the water/salt-soluble fraction was confirmed (data not shown) by enzyme assay.

In vitro pepsin resistance of amylase enriched fractions was investigated by a newly developed method. Proteins were transblotted on the PVDF membrane after their SDS-PAGE separation and digested on the membrane in vitro by pepsin. The IgE reactive pepsin-resistant bands were identified by using wheat sensitive human sera and TrimW specific rabbit IgG (Figure 3). The polyclonal antibody identified

Table 1. Wheat proteins identified by LC-MS/MS combined with Uniprot trembl database.

PAGE band	Protein name	Taxonomy	Mw (Da)	Number of unique peptides	Sequence coverage (%)	Primary accession number	Additional accession numbers
K1	Serpin-Z1A	<i>Triticum aestivum</i>	43,101.3	3	8.79	Q41593	Q41593
	Tritin	<i>Triticum aestivum</i>	29,595.2	2	9.45	Q07810	Q07810, Q7M1Z3
	Xylanase inhibitor protein 1	<i>Triticum aestivum</i>	33,257.3	4	13.20	Q8L5C6	Q8L5C6
K2	Endogenous alpha-amylase/subtilisin inhibitor	<i>Triticum aestivum</i>	19,614.9	5	38.30	P16347	P16347
K3	Dimeric alpha-amylase inhibitor (Fragment)	<i>Triticum turgidum</i> subsp. dicoccoides	13,162.9	4	47.60	A4GFN8	A4GFN8
	Monomeric alpha-amylase inhibitor (Fragment)	<i>Triticum aestivum</i>	13,136.5	6	54.50	A4ZIW9	A4ZIU3, A4ZIU7, A4ZIV8, A4ZIW9
K4	Dimeric alpha-amylase inhibitor (Fragment)	<i>Triticum turgidum</i> subsp. dicoccoides	13,162.9	5	59.70	A4GFN8	A4GFN8
	Monomeric alpha-amylase inhibitor (Fragment)	<i>Triticum aestivum</i>	13,136.5	6	55.40	A4ZIW9	A4ZIU3, A4ZIU7, A4ZIV8, A4ZIW9
	Wheatwin-1	<i>Triticum aestivum</i>	15,616.2	2	11.00	O64392	O64392
K5	Endosperm transfer cell specific PR60	<i>Triticum aestivum</i>	11,889.2	2	19.60	B2C4K0	B2C4K0
	Monomeric alpha-amylase inhibitor (Fragment)	<i>Triticum aestivum</i>	13,136.5	2	19.00	A4ZIW9	A4ZIU3, A4ZIU7, A4ZIV8, A4ZIW9
	Non-specific lipid-transfer protein (Fragment)	<i>Triticum aestivum</i>	11,880.4	4	32.70	P24296	P24296
	Non-specific lipid-transfer protein 2G	<i>Triticum aestivum</i>	6960.7	4	92.50	P82900	P82900
K7	Alpha-amylase/trypsin inhibitor CM3	<i>Triticum aestivum</i>	18,203.2	2	19.00	P17314	P17314
	Beta-amylase	<i>Hordeum vulgare</i>	59,629.8	6	14.20	P16098	P16098

Table 1 (Continued)

PAGE band	Protein name	Taxonomy	Mw (Da)	Number of unique peptides	Sequence coverage (%)	Primary accession number	Additional accession numbers
K8	Globulin 3	<i>Triticum aestivum</i>	66,330.4	3	4.76	B7U6L4	B7U6L4
	Phosphoglycerate kinase, cytosolic	<i>Triticum aestivum</i>	42,105.5	4	12.70	P12783	P12783
	Serpin-Z1A	<i>Triticum aestivum</i>	43,101.3	3	5.78	Q41593	Q41593
	Serpin-Z2A	<i>Triticum aestivum</i>	43,294.5	2	9.30	Q9ST57	Q9ST57
K10	Serpin-Z2B	<i>Triticum aestivum</i>	42,964.1	3	6.53	P93692	P93692
	Alpha-amylase/trypsin inhibitor CM3	<i>Triticum aestivum</i>	18,203.2	3	28.00	P17314	P17314
	Globulin 3	<i>Triticum aestivum</i>	66,330.4	2	2.89	B7U6L4	B7U6L4
	Superoxide dismutase (Fragment)	<i>Triticum aestivum</i>	19,302.1	3	23.00	A3FKE5	A3FKE5
K12	Serpin-Z1A	<i>Triticum aestivum</i>	43,101.3	5	12.30	Q41593	Q41593
	Serpin-Z1C	<i>Triticum aestivum</i>	42,864.4	2	16.30	Q9ST58	Q9ST58
	Serpin-Z2B	<i>Triticum aestivum</i>	42,964.1	7	21.60	P93692	P93692
K13	Alpha-amylase/trypsin inhibitor CM3	<i>Triticum aestivum</i>	18,203.2	2	19.00	P17314	P17314
	Serpin-Z1A	<i>Triticum aestivum</i>	43,101.3	11	35.40	Q41593	Q41593
	Serpin-Z1C	<i>Triticum aestivum</i>	42,864.4	5	34.90	Q9ST58	Q9ST58
	Serpin-Z2B	<i>Triticum aestivum</i>	42,964.1	5	17.80	P93692	P93692
K14	Alpha-amylase/trypsin inhibitor CM16	<i>Triticum aestivum</i>	15,764.1	2	11.90	P16159	P16159
	Alpha-amylase/trypsin inhibitor CM2	<i>Triticum aestivum</i>	15,441.3	4	31.70	P16851	P16851
	Alpha-amylase/trypsin inhibitor CM3	<i>Triticum aestivum</i>	18,203.2	7	64.30	P17314	P17314
	Dimeric alpha-amylase inhibitor (Fragment)	<i>Triticum turgidum</i> subsp. <i>dicoccoides</i>	13,162.9	4	47.60	A4GFN8	A4GFN8

Table 1 (Continued)

PAGE band	Protein name	Taxonomy	Mw (Da)	Number of unique peptides	Sequence coverage (%)	Primary accession number	Additional accession numbers
K15	Alpha-amylase/trypsin inhibitor CM16	<i>Triticum aestivum</i>	15,764.1	3	23.10	P16159	P16159
	Alpha-amylase/trypsin inhibitor CM2	<i>Triticum aestivum</i>	15,441.3	2	13.80	P16851	P16851
	Alpha-amylase/trypsin inhibitor CM3	<i>Triticum aestivum</i>	18,203.2	5	42.30	P17314	P17314
	Dimeric alpha-amylase inhibitor (Fragment)	<i>Triticum turgidum</i> subsp. dicoccoides	13,162.9	4	44.40	A4GFN8	A4GFN8
	Glutenin, high molecular weight subunit 12	<i>Triticum aestivum</i>	69,604.9	2	3.24	P08488	P08488, P10387
K16	0.19 dimeric alpha-amylase inhibitor (Fragment)	<i>Aegilops tauschii</i>	13,300.8	2	32.30	Q5UHH1	Q5UHH1
	Alpha-amylase/trypsin inhibitor CM16	<i>Triticum aestivum</i>	15,764.1	2	11.90	P16159	P16159
	Alpha-amylase/trypsin inhibitor CM3	<i>Triticum aestivum</i>	18,203.2	3	28.60	P17314	P17314
	Avenin-like protein	<i>Triticum aestivum</i>	33,013.5	2	6.53	A5A4L4	A5A4L4, A5A4L5, A7XUL9, A7XUM1, A7XUM9, A7XUN5, A7XUN7, A7XUP2, A7XUP6, A7XUP8, A7XUQ1, A7XUQ5, A7XUQ7, B8YG97, D0EWS4, Q2A777, Q2A778, Q2A779, Q2A783
	Dimeric alpha-amylase inhibitor	<i>Eremopyrum bonaepartis</i>	15,178.9	2	38.30	C3VWV4	C3VWV4

Table 1 (Continued)

PAGE band	Protein name	Taxonomy	Mw (Da)	Number of unique peptides	Sequence coverage (%)	Primary accession number	Additional accession numbers
	Dimeric alpha-amylase inhibitor (Fragment)	<i>Triticum turgidum</i> subsp. dicoccoides	13,162.9	2	19.40	A4GFN8	A4GFN8
	Glutenin, high molecular weight subunit 12	<i>Triticum aestivum</i>	69,604.9	3	4.63	P08488	P08488, P10387
	Trypsin/alpha-amylase inhibitor CMX2	<i>Triticum aestivum</i>	13,813.4	2	16.50	Q43691	Q43691, Q43723
	Ubiquitin	<i>Triticum aestivum</i>	8,507.2	2	32.90	P69326	P0C072, P69308, P69310, P69312, P69314, P69316, P69318, P69320, P69322, P69324, P69326

Table 2. Identified proteins non-cross-reactive with wheat proteins by LC-MS/MS combined with Uniprot trembl database.

PAGE band	Protein name	Taxonomy	Mw (Da)	Number of unique peptides	Sequence coverage (%)	Primary accession number	Additional accession numbers
K17	Agglutinin	<i>Amaranthus hypochondriacus</i>	34,940.6	5	26.00	Q38719	Q38719, Q8S390
K18	13S globulin seed storage protein 2	<i>Fagopyrum esculentum</i>	57,024.8	3	15.90	O23880	O23880
	13S globulin seed storage protein 3	<i>Fagopyrum esculentum</i>	61,145.1	10	24.20	Q9XFM4	Q9XFM4
K19	1-Cys peroxiredoxin PER1	<i>Zea mays</i>	24,887.5	3	13.10	A2SZW8	A2SZW8
	40S ribosomal protein S3	<i>Zea mays</i>	25,416.9	2	11.80	B4G0E1	A2XIT7, A2YNK7, B4G0E1, B6TB97, B6TM40, C0HE21, C0P4F7, C5YHF8, Q10HT1, Q6YTY2, Q75G91, Q94LL1

Table 2 (Continued)

PAGE band	Protein name	Taxonomy	Mw (Da)	Number of unique peptides	Sequence coverage (%)	Primary accession number	Additional accession numbers
	Endochitinase A	<i>Zea mays</i>	29,578.3	4	18.20	B6TEL0	B6TEL0, B8QUV9, B8QUW0, B8QUX0, B8QUY3, B8QUY5, B8QV01, Q6JBK4
	Endochitinase B (Fragment)	<i>Zea mays</i>	28,146.9	2	14.90	P29023	P29023
	Globulin-1 S allele	<i>Zea mays</i>	49,945.5	6	17.30	B6UGJ0	B6UGJ0, Q7M1Z8
	Glutathione S-transferase GSTF2	<i>Zea mays</i>	23,789.8	2	9.35	B6SKA0	B6SKA0, B6U1A7, C5XKV8
	Late embryogenesis abundant protein, group 3	<i>Zea mays</i>	22,740.4	2	10.90	Q42376	Q42376
	Lipoprotein	<i>Zea mays</i>	26,205.6	2	11.30	B4FFZ9	B4FFZ9, B6UGJ9
	Triosephosphate isomerase, cytosolic	<i>Zea mays</i>	27,005.7	5	25.30	P12863	P12863
K20	Alanine aminotransferase	<i>Oryza sativa</i> subsp. japonica	52,577.0	2	4.14	Q338N8	B8BGM4, Q338N8, Q94HC5, Q9S768
	ATP synthase subunit beta, mitochondrial	<i>Oryza sativa</i> subsp. japonica	60,241.7	2	4.98	P17614	P17614, P29685
	Cupin family protein, expressed	<i>Oryza sativa</i> subsp. japonica	63,409.3	5	12.50	Q75GX9	Q75GX9
	Enolase	<i>Oryza sativa</i> subsp. japonica	47,955.0	5	20.00	Q42971	Q42971
	Putative uncharacterised protein	<i>Oryza sativa</i> subsp. indica	52,041.9	4	13.40	B8AL97	B8AL97, Q852L2
	UDP-glucose pyrophosphorylase	<i>Oryza sativa</i> subsp. indica	51,635.9	6	17.10	A3QQQ3	A3QQQ3, A4KC21, Q93X08

IgE reactive bands in samples at 45 kDa in conformity with the molecular weight of serpins, and at 16 kDa according to that of AAIIs (Figure 3b). While strong bands have been observed even after 30-minute-digestion, 60-minute-digestion gave visible, but only slight bands. The wheat sensitive human sera recognised resistant immune-

Table 3. Identified cross-reactive proteins with wheat proteins by LC-MS/MS combined with Uniprot trembl database.

PAGE band	Protein name	Taxonomy	Mw (Da)	Number of unique peptides	Sequence coverage (%)	Primary accession number	Additional accession numbers
K21	Beta-amylase	<i>Hordeum vulgare</i>	59629.8	2	3.55	P16098	P16098
	Beta-amylase (Fragment)	<i>Secale cereale</i>	24,331.0	2	21.20	P30271	P30271
	Enolase	<i>Oryza sativa</i> subsp. <i>japonica</i>	47,955.0	2	7.40	Q42971	Q42971
	Globulin 3	<i>Triticum aestivum</i>	66,330.4	2	4.25	B7U6L4	B7U6L4
K22	Beta-amylase (Fragment)	<i>Secale cereale</i>	24,331.0	2	14.40	P30271	P30271
	Phosphoglycerate kinase, cytosolic	<i>Triticum aestivum</i>	42,105.5	2	7.48	P12783	P12783
	Serpin-Z1A	<i>Triticum aestivum</i>	43,101.3	4	13.60	Q41593	Q41593
K23	Globulin 3	<i>Triticum aestivum</i>	66,330.4	2	3.23	B7U6L4	B7U6L4
	Serpin-Z1A	<i>Triticum aestivum</i>	43,101.3	3	7.54	Q41593	Q41593
K25	Putative uncharacterised protein Sb01g039390 (HSP70)	<i>Sorghum bicolor</i>	71,442.6	6	11.20	C5WPE1	C5WPE1
K26	Barley protein Z homolog (Fragment)	<i>Avena fatua</i>	31,095.8	4	12.10	Q9XET7	Q9XET7
K28	Alpha-1,4-glucan-protein synthase [UDP-forming]	<i>Phoenix dactylifera</i>	6,829.1	2	36.70	P85413	P85413
	Aspartate aminotransferase, cytoplasmic	<i>Oryza sativa</i> subsp. <i>japonica</i>	44,490.0	2	5.90	P37833	P37833
	Fructose-bisphosphate aldolase	<i>Secale cereale</i>	38,707.1	3	10.10	C1J958	A2XUB6, C1J958, C5XRQ7, Q10A30, Q40676
	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	<i>Antirrhinum majus</i>	36,667.1	3	11.90	P25861	P25861

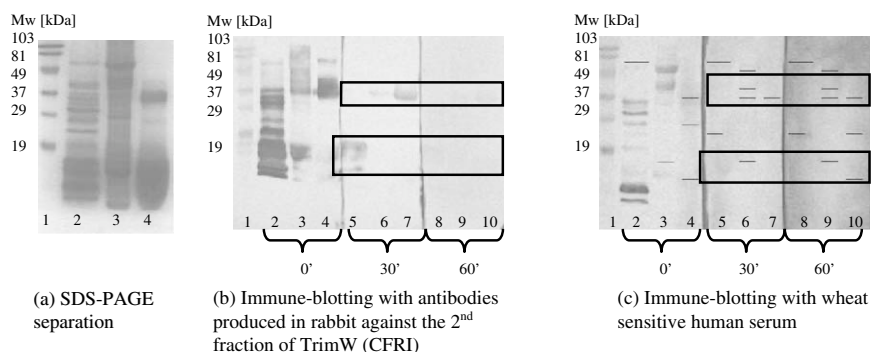


Figure 3. *In vitro* investigation of pepsin digestion on PVDF membrane-blotted wheat proteins. 1. Protein marker (Mw.); 2. *T. aestivum*, GK Cipó, 2004 – 1st fraction, non-digested; 3. *T. aestivum*, GK Cipó, 2004 – 2nd fraction, non-digested; 4. TrimW-2nd frakció, non-digested; 5. *T. aestivum*, GK Cipó, 2004 – 1st fraction, digested for 30 min.; 6. *T. aestivum*, GK Cipó, 2004 – 2nd fraction, digested for 30 min.; 7. TrimW-2nd fraction, digested for 30 min.; 8. *T. aestivum*, GK Cipó, 2004 – 1st fraction, digested for 60 min.; 9. *T. aestivum*, GK Cipó, 2004 – 2nd fraction, digested for 60 min.; 10. TrimW-2nd fraction, digested for 60 min.

reactive bands at 16, 18, 26, 37, 41, 50, 67 kDa against pepsin digestion, where the time of digestion did not influence the intensity of the protein bands (Figure 3c).

With such studies it was confirmed that allergens are, in fact, resistant towards pepsin digestion. It was also evident that the pepsin-resistant and strongly reactive proteins towards human sera were mostly serpins (45 kDa) and AAIIs (16 kDa).

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