

Antibody reactivity in patients with IgE-mediated wheat allergy to various subunits and fractions of gluten and non-gluten proteins from ω -gliadin-free wheat genotypes

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Abstract

Introduction and objective. Gluten proteins (gliadins and glutenins) are polymorphic wheat storage proteins of allergenic properties. Significant differences in chemical composition between both protein groups allow to expect highly specific immunological response of individual subunits and fractions in reactions with IgE sera of people allergic to wheat. The aim of these studies was to identify and characterize the most allergenic gluten proteins (GP) and nongluten proteins (NGP) occurred in two closely related wheat hybrid genotypes.

Materials and method. 3xC and 3xN wheat hybrids, which differ strongly in regard of gliadin composition, were analyzed. Seven people manifesting different symptoms of wheat allergy donated sera for the experiment. The technique of immunoblotting after SDS-PAGE was used for identification of allergenic subunits and fractions among GP and NGP. Immunologically active protein bands were visualized by chemiluminescence.

Results. Great variation of immunodetection spectra was observed. Results of immunoblotting showed LMW glutenins to be of highest, gliadins of medium, while NGP of lowest allergenicity for selected patients. The 43-kDa and 47-kDa LMW glutenin subunits, 40-kDa and 43-kDa γ -gliadin fractions and 49-kDa NGP can be considered as the most immunoreactive among all protein bands separated by SDS-PAGE.

Conclusion. The observed differentiation of immunodetection spectra allows to model highly specific IgE-binding profiles of allergenic wheat proteins attributed to individual patients with symptoms of gluten intolerance. Highly immunoreactive subunits and fractions among GP and NGP were identified. The observed immunoreactivity of 49 kDa NGP is worth to emphasize, as it has never been reported as wheat allergenic protein before.

Key words

gliadins, glutenins, immunoblotting, food allergy, wheat

INTRODUCTION

In medical sciences gluten is considered as proteinaceous substance of toxic and allergenic properties that is able to trigger celiac and food allergies in people sensitized to wheat proteins. Gluten is composed of hundreds of polymorphic proteins expressed in the endosperm of wheat kernels as storage proteins [1, 2]. More than 80% of these proteins belong to gliadins and glutenins, defined as gluten proteins (GP) [3, 4]. Gliadins and glutenins of individual wheat genotypes may be separated into dozens of protein bands using one-dimensional electrophoretic techniques (A-PAGE or SDS-PAGE), whereas hundreds of spots may be developed using 2D electrophoresis (IEF/SDS-PAGE) [5, 6]. Traditionally, gliadins are divided into α , β , γ , ω -5 and ω -1.2 subclasses based on decreased electrophoretic mobility in A-PAGE conditions. In turn, glutenins are composed of high molecular weight

(HMW) and low molecular weight (LMW) subunits based on SDS-PAGE separations [7].

Glutenins are polymeric proteins. The C- and N-terminal sequences in glutenin polypeptides contain from one to five cysteine residues that bind individual subunits via intermolecular disulphide bonds (SS), forming large polymeric complexes [8]. Gliadins belonging to α , β and γ groups also contain cysteines capable of forming SS bonds, however, these disulphides are frequently of intramolecular nature. In consequence, gliadins usually occur as monomeric proteins [9]. Gliadins and glutenins differ also with respect to the number, amino acid composition and localization of short repetitive sequences within polypeptides. The most typical motives are: PQQPFP and PQQPY in α -gliadins, QQQP in γ -gliadins and LMW glutenins, PQQPFPQQ in ω -gliadins and PGQGQQ in HMW glutenins [8, 10–12].

Disulphide bonds and repetitive sequences are possibly the most important details influencing immunoreactive properties of GP. SS bonds determine the spatial conformation of the proteins while repetitive sequences may act as IgE binding epitopes (like QQQP pentapeptide) [8, 9, 11, 13, 14]. In consequence, some epitopes can be hidden in the interior,

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while the others exposed on the outer surface of the protein molecules. This may facilitate or aggravate interactions with hyper-variable regions of sIgE antibodies in sera of allergic people, affecting immunoreactive properties of proteins [15].

A range of allergic diseases caused by GP have been diagnosed in the last three decades. One of the important findings in allergology was to prove that ω -5 gliadins are the main allergens involved in wheat-dependent exercise-induced anaphylaxis (WDEIA) – the most dangerous, life threatening form of wheat allergy [16–18]. Omega-5 gliadins may also trigger other wheat allergies, described as exercise-induced wheat allergy (EIWA) [18]. In turn, ω -1.2 gliadins were evidenced to be allergenic for patients with atopic dermatitis [19]. LMW glutenins play an important role in the development of immediate hypersensitivity to hydrolyzed wheat proteins (IHHWPs) baker's asthma, atopic dermatitis and EIWA [18, 20]. Battais et al. showed elevated levels of IgE antibodies against all GP in the sera of 28 patients with atopic dermatitis, asthma, urticaria and eczema, which suggested that these proteins were important factors contributing to disease development [21, 22].

Another important, proteinaceous components of wheat kernels are nongluten proteins (NGP). Most of them belong to water/salt soluble albumin and globulin groups. The most allergenic NGP are members of the α -amylase/trypsin inhibitors family. In cereals they usually occur as tetrameric or dimeric protein molecules. Low molecular weight subunits (about 12 kDa) of these proteins – CM2, CM3 and CM16 – known to be major allergens in baker's asthma, have been extensively investigated [23, 24]. Salt soluble NGP of higher molecular weight (20 kDa–60 kDa) were the objects of this study.

The high polymorphism of gliadins and glutenins, the complex nature of albumins and globulins and a variety of disease symptoms in wheat allergies allow to expect a highly specific immunological response to GP and NGP. One can hypothesize that this specificity may occur not only at the level of protein classes or subclasses, but also at the level of individual subunits and fractions. It would be important to demonstrate which of them are highly allergenic, which are of low allergenicity, or not allergenic at all. Previously we have developed and analyzed hypoallergenic wheat genotypes lacking all ω -gliadins (3xN), and control genotypes containing all ω -gliadin fractions (3xC) [25]. In the present study we used these genotypes as a model system to investigate immunoreactivity of individual subunits and fractions of GP and NGP.

OBJECTIVES

The general purpose of this work was to find out whether wheat kernel proteins – comprising both gluten and nongluten proteins – of predicted combination of subunits and fractions, may sensitize wheat allergic persons in distinct manners, causing various symptoms of wheat allergies.

MATERIALS AND METHOD

Plant material. 3xC and 3xN winter wheat hybrid genotypes were analyzed. Both wheats were developed using traditional plant breeding methods based on cross combinations and

selection of desirable individuals. Each of parental forms used for crossing donated single null alleles (inactive gene variants) in ω -gliadin coding loci (*Gli A1*, *Gli B1* and *Gli D1*) localized on chromosomes 1A, 1B and 1D. As result of genetical recombinations the 3xN wheat containing all three null alleles and devoid of all ω -gliadins was developed. In contrast, the 3xC control line contained the full set of ω -gliadins. In 3xN considerable decreasing of gliadin allergenic properties and improving of wheat technological properties as compared to 3xC was demonstrated. A complete characteristics of wheat kernel proteins' composition by A-PAGE, SDS-PAGE and RP-HPLC, technological properties and gliadins' immunoreactivity in 3xC and 3xN have been described in one of our previous works [25]. In the present work both wheat genotypes were used as a model system for the research on wheat allergy.

Patients. Seven individuals manifesting different symptoms of wheat allergy and different sIgE levels in response to gluten were selected from among patients treated at the Department of Clinical and Environmental Allergology, Jagiellonian University, Collegium Medicum (Krakow, Poland) (table 1).

Extraction and separation of wheat proteins. Kernel samples from 3xC and 3xN wheats (200 g each) were milled using a Quadrumat Junior (Brabender, Germany) laboratory mill. 400 μ l of 0.15 M NaCl was added to 40- μ g flour samples to remove NGP. In order to improve the selectivity of NGP extraction 0.15 M NaCl (instead 0.5 M NaCl) was used. The obtained suspension was centrifuged (1000 rpm, 10 min) and supernatants were stored in Eppendorf tubes. Pellets were incubated with 400 μ l of 70% EtOH for two hours to extract gliadins (the obtained gliadins were analyzed in both the reduced and non-reduced state). After centrifugation, supernatants were stored, while the pellets were treated overnight with glutenin extraction buffer composed of 6 M urea, 2.5% SDS and 1.5% mercaptoethanol and centrifuged again. The resulting extract contained HMW and LMW glutenins.

Composition of wheat kernel proteins was determined by SDS-PAGE. Proteins were separated in a Mini Protean II Cell using Mini Protean TGX Precast Gels, 10% Resolving Gel (8.6 cm \times 7.2 cm) in ten-fold diluted Tris/Glycine/SDS concentrated buffer (BioRad, USA). During each electrophoretic run, four protein extracts: glutenins, native and reduced gliadins and NGP were separated on two gel slabs simultaneously. Electrophoresis was carried out for 50 min at a constant voltage of 200 V, while the current varied during the run. After electrophoresis, one of the two gel slabs was stained in Coomassie Brilliant Blue (0.02% R 250) dye solution to visualize all protein subunits and fractions, while the second one was used for blotting and immunodetection of separated proteins.

Blotting and detection of immunoreactive proteins. Proteins from the gel slabs were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, USA) of 0.45- μ m thickness, fitted to the dimensions of the gel slab. Blotting was performed using a Transblot SD apparatus (BioRad, USA) in semidry conditions according to Curioni et al. (1991) [26] for 1 hour at 100 V. Detection of immunoreactive protein subunits and fractions was performed according to the procedure described in our

previous work with several modifications [27]. Membranes after electrotransfer were placed in a blotting container and blocked for 1 hour in 3% defatted, commercial milk with 1% bovine serum albumin in PBST solution. PBST was prepared from ready-to-use pills (Sigma, USA) according to the manufacturer's instruction with the addition of 100 µl of Tween. Immediately after removing the blocking agent, sera of individual patients diluted 1:10 in PBST were added to the container with PVDF membrane and gently shaken overnight. After incubation, membranes were washed five times with PBST for about 40 min and incubated again with secondary antibodies – anti-human IgE conjugated with HRP (Sigma, USA) diluted 1:1000 in PBST. Immunoreactive proteins were detected by incubation with a chemiluminescence substrate (BioRad, USA) for 5 min and visualized in a Chemi Doc XRS+ molecular imager (BioRad, USA). Image analysis and calculation of molecular masses were performed using Image Lab Software (BioRad, USA).

RESULTS

Great differentiation of immunoblotting patterns between individual patients and wheat genotypes was observed. Sera of four persons showed positive reactions with GP (glutenins and reduced gliadins), but not with NGP. Sera of the remaining three subjects recognized both GP and NGP. However, no immunoreaction was observed for non-reduced gliadins.

Three and four immunoreactive bands were identified among 3xC and 3xN GP, respectively, probed with the serum of patient 1 (Figure 1). In the 3xC wheat, one LMW glutenin of MW=32 kDa and one HMW glutenin of MW=101 kDa (corresponding to Dx2 subunit) were clearly visible. However, in the 3xN wheat, two LMW glutenins (43 and 36 kDa) and one HMW glutenin of 101 kDa were detected. In turn, one protein band of MW=30 kDa was detected among the reduced gliadins in both 3xC and 3xN wheat genotypes.

More immunoreactive proteins were recorded on the immunoblot of patient 2 (Figure 2). Four and five subunits of LMW glutenins in 3xC and 3xN, respectively, showed clear chemiluminescence signals. Their molecular weights

ranged from 32 to 50 kDa. Two immunoreactive fractions of reduced gliadins were identified in the 3xC and 3xN wheats: a 32-kDa gliadin band was common for both genotypes, while the second one had 36 kDa in 3xC and 40 kDa in 3xN.

Although the immunodetection quality for sera 3 and 4 was poorer, several immunoreactive proteins could be identified. LMW subunits of molecular weights: 47 kDa, 43 kDa and 40 kDa were detected in both 3xC and 3xN lines probed with the serum of patient 3 (Figure 3). This triplet in 3xN was accompanied by one additional 38-kDa band. Among the reduced gliadins, three immunoreactive bands (43, 38 and 30 kDa) were identified in 3xN, however, in 3xC these were spots rather than clear band that were acquired. This was particularly the case for proteins of molecular weight ranging from 30 to 38 kDa. In contrast to other immunoblotting patterns obtained in this study, immunoblots of the 3xC wheat detected a 60-kDa ω-5 gliadin band both in the reduced and non-reduced extracts.

Serum of patient 4 reacted with a number of glutenin subunits (30–88 kDa) and reduced gliadins (30–47 kDa). A wide spectrum of immunoreactive bands comprised almost all of the 3xC and 3xN wheat proteins (Figure 4).

Three patients potentially allergic to GP as well as NGP demonstrated specific patterns of immunoreactivity. Nine and eleven glutenin subunits in the 3xC and 3xN wheat lines, respectively, were immunodetected in the sample with the serum of patient 5 (Figure 5). The expressed glutenin bands were well separated and of medium intensity. The LMW subunit of 40 kDa in the 3xC wheat was the only one showing slightly stronger luminescence signal as compared to other immunoreactive proteins. Reduced gliadins, in turn, were represented as faint blurry spots ranging in molecular weight from 30 to 43 kDa. In contrast, a single sharp band of 49 kDa was observed among NGP in both wheats.

Numerous immunoreactive glutenin bands probed with the serum of patient 6 were observed. A single band of 47 kDa showed stronger luminescence as compared to other HMW and LMW subunits in the 3xC and 3xN wheats (Figure 6). Among the reduced gliadins, one and two immunoreactive bands were detected in 3xC and 3xN, respectively. Contrary to patient 5, these signals were weaker, but the bands were

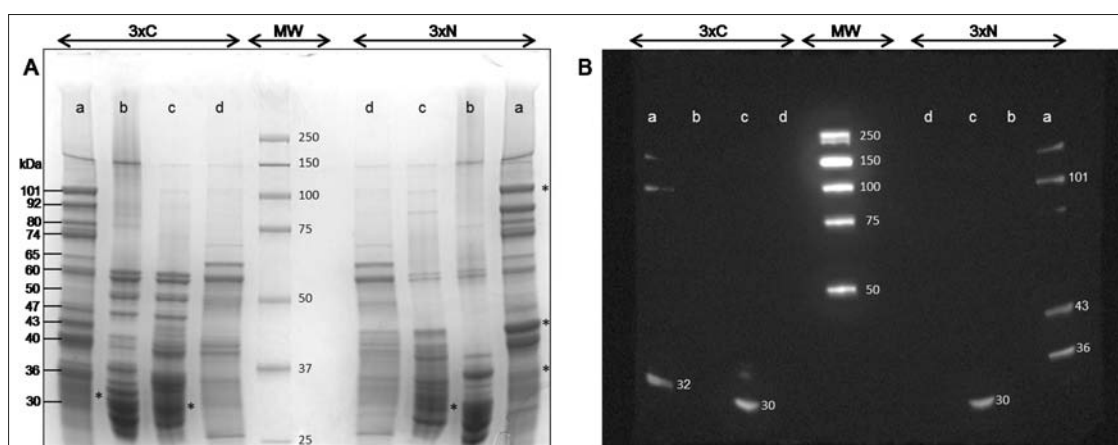


Figure 1. Wheat kernel proteins (glutenins, gliadins and salt soluble proteins) extracted from 3xC and 3xN wheats, separated by SDS-PAGE (A) and immunoblotted with the IgE sera of patient 1 (B). Immunoreactive subunits and fractions are marked by asterisk (on the electrophoregram) and by molecular mass values (on the immunoblot) on the right hand sides of each run. Molecular masses of the reference bands for HMW and LMW glutenin subunits are given on the left hand side of the run „a” (a – HMW and LMW glutenin subunits, b – non-reduced gliadins, c – gliadins reduced by mercaptoethanol, d – salt soluble proteins)

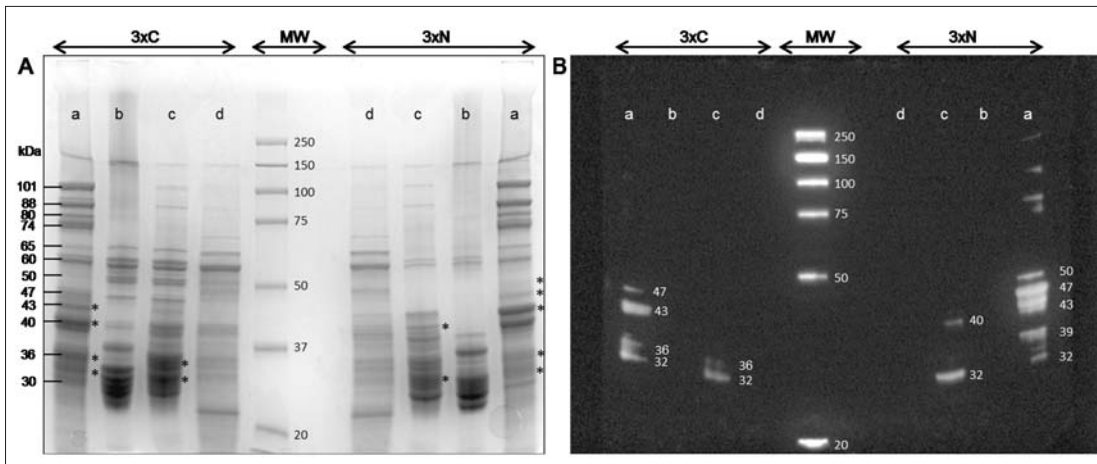


Figure 2. Patient 2. Electrophoretic (A) and immunoblotting (B) data as on Figure 1

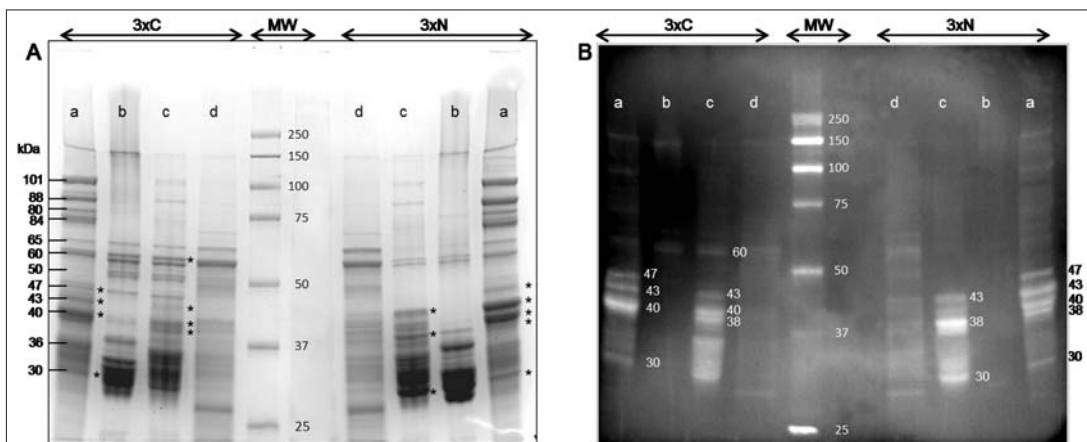


Figure 3. Patient 3. Electrophoretic (A) and immunoblotting (B) data as on Figure 1

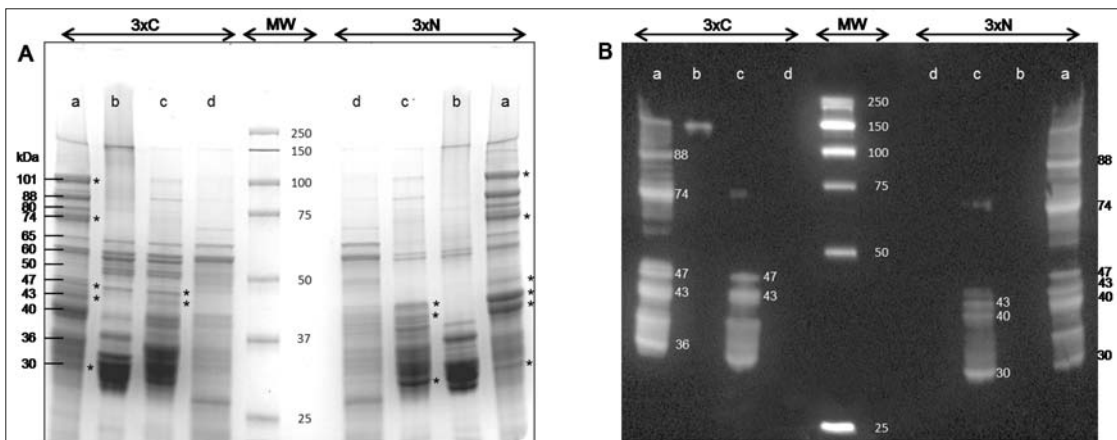


Figure 4. Patient 4. Electrophoretic (A) and immunoblotting (B) data as on Figure 1

clear and sharp. NGP protein extracts gave a single distinct 49-kDa band in both varieties. Moreover, in 3xC this protein was accompanied by an additional 36-kDa band.

The highest number of immunoreactive proteins was observed on the immunoblot of patient 7 in all protein groups (Figure 7). Several LMW subunits could be classified as highly immunoreactive. In the 3xC variety these were bands of 47, 43, 42 and 40 kDa, and 47-kDa and 43-kDa proteins in the 3xN wheat. It is worth emphasizing that the 43-kDa band in the 3xN wheat extract had the highest luminescence intensity of all proteins immunodetected in this study. The 43-kDa

gliadin band of 3xN wheat was also clear and markedly distinct from other smeary overlapping and poorly stained fractions. A wide spectrum of immunoreactive NGP bands (ranging from 14 to 65 kDa) was identified in both 3xC and 3xN wheats. However, they were poorly stained and frequently overlapped each other. The only exception was a 43-kDa protein in the extract of 3xN wheat that was also clearly stood out from other NGP.

Based on the frequency of immunoreactive bands, several GP and NGP can be considered as important allergens for the patients examined. The highest number of detected proteins

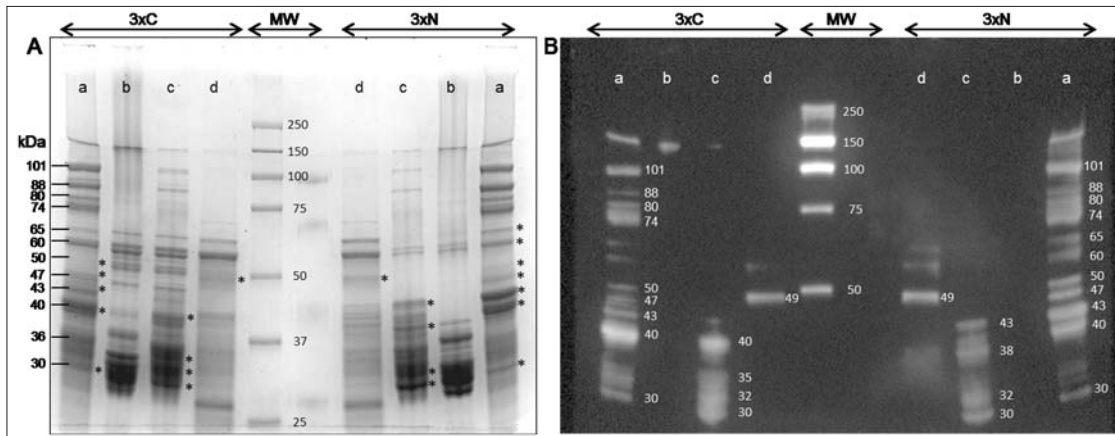


Figure 5. Patient 5. Electrophoretic (A) and immunoblotting (B) data as on Figure 1

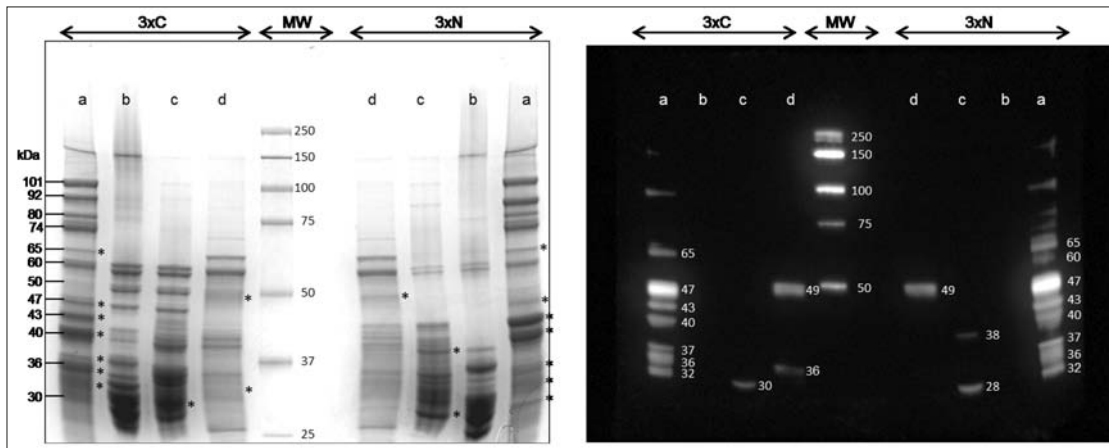


Figure 6. Patient 6. Electrophoretic (A) and immunoblotting (B) data as on Figure 1

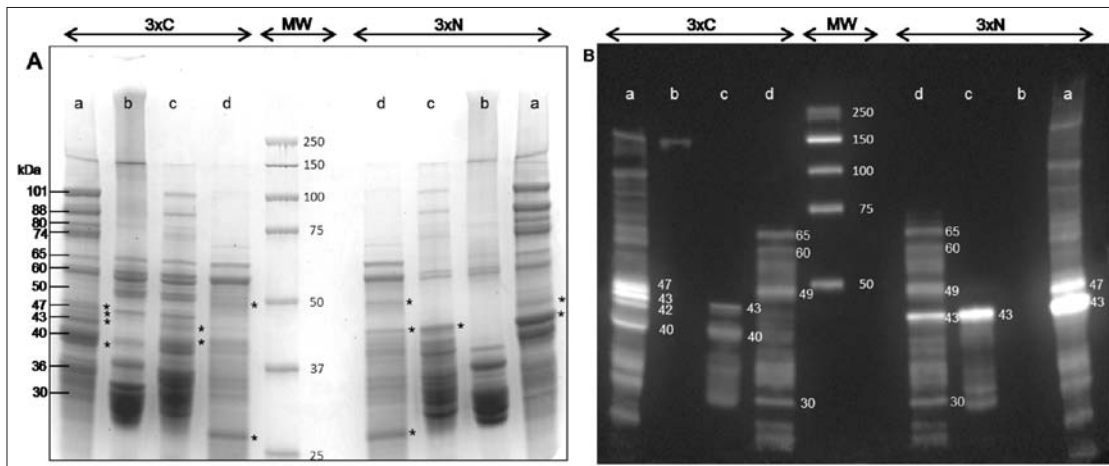


Figure 7. Patient 7. Electrophoretic (A) and immunoblotting (B) data as on Figure 1

was observed among glutenins, especially among the LMW subunits. A total of 13 LMW glutenin bands in 3xC and 16 in the 3xN wheat were classified as immunoreactive proteins (Table 1). In the 3xC wheat, 43-kDa and 47-kDa subunits were detected in almost all patients studied. The remaining 32-kDa, 36-kDa and 40-kDa subunits were immunoreactive in about half of the subjects included in the experiments. HMW glutenins of molecular weights 74 and 88 kDa, corresponding to Dy12 and Bx7 subunits, respectively, were detected in the sera of two patients. Other glutenins showed immunoreactivity with sera of individual patients.

Ten gliadin fractions specific for the 3xC wheat and five gliadins specific for 3xN were recognized as immunoreactive in the current study (Table 2). The most frequently immunodetected gliadins of molecular weights 30, 40, and 43 kDa were recorded in approximately half of the study patients. The remaining fractions, including 47-kDa γ -gliadin and 60-kDa ω -gliadin, from the 3xC variety were immunoreactive in single patients, however, no immunodetection of these bands was observed with 3xN extracts.

Among six immunodetected NGP, only one fraction of MW=49 kDa was recognized by the sera of all three patients

Table 1. Clinical data of seven wheat-allergic patients

Patient No.	Sex	Age	Profession	Wheat induced symptoms
1	M	67	baker – confectioner	rhinitis, asthma
2	M	53	house keeper	urticaria, angioedema
3	M	57	policeman	anaphylactic reactions (urticaria, tongueangioedema and anaphylactic shock)
4	M	34	baker – confectioner	rhinitis, asthma, allergic contact dermatitis
5	F	52	book-keeper	asthma, rhinitis
6	M	36	baker – confectioner	asthma
7	M	44	desktop publisher	asthma, atopic dermatitis

Table 2. Frequency distribution of immunoreactive HMW and LMW glutenin subunits in the analyzed group of wheat-allergic patients

MW (kDa)	3xC		MW (kDa)	3xN	
	NP	%all		NP	%all
43	6	86	43	7	100
47	6	86	47	6	86
40	4	57	40	4	57
32	3	43	50	2	29
36	3	43	36	2	29
74	2	29	32	2	29
88	2	29	30	2	29
30	1	14	88	2	29
37	1	14	74	2	29
50	1	14	65	2	29
65	1	14	60	2	29
80	1	14	101	1	14
101	1	14	39	1	14
38	0	0	38	1	14
39	0	0	80	1	14
60	0	0	37	1	14

NP – number of patients sensitized to a protein of predicted molecular weight
all – percentage in relation to all patients participating in the experiment

sensitized to NGP (Table 3). The remaining NGP were immunodetected by individual patients.

Table 3. Frequency distribution of immunoreactive reduced gliadin fractions in the analyzed group of wheat-allergic patients

MW (kDa)	3xC		MW (kDa)	3xN	
	NP	%all		NP	%all
30	3	43	30	5	71
40	3	43	43	4	57
43	3	43	38	3	43
32	2	29	32	2	29
28	1	14	40	2	29
35	1	14	28	0	0
36	1	14	35	0	0
38	1	14	36	0	0
47	1	14	47	0	0
60	1	14	60	0	0

NP – number of patients sensitized to a protein of predicted molecular weight
all – percentage in relation to all patients participating in the experiment

Table 4. Frequency distribution of immunoreactive salt soluble proteins in the analyzed group of wheat-allergic patients

MW (kDa)	3xC			MW (kDa)	3xN		
	NP	%s.s.	%all		NP	%s.s.	%all
49	3	100	43	49	3	100	43
30	1	33	14	30	1	33	14
36	1	33	14	43	1	33	14
60	1	33	14	60	1	33	14
65	1	33	14	65	1	33	14
43	0	0	0	36	0	0	0

NP – number of patients sensitized to a protein of predicted molecular weight; s.s. – percentage in relation to patients sensitized to salt soluble proteins; all – percentage in relation to all patients participating in the experiment

DISCUSSION

In the present study immunoreactive properties of individual subunits and fractions of GP and NGP in two closely related wheat hybrid genotypes 3xC and 3xN were analyzed. Immunoblotting technique was used to investigate whether, and to what extent, patterns of immunoreactive proteins were specific for individual wheat allergic patients. The analyzed wheat hybrids were immuno-positive for a number of bands representing α , β , γ gliadins, LMW glutenins and NGP. These results have confirmed the immunoreactivity of α , β and γ gliadins, previously reported in our study that tested purified gliadin fractions by ELISA [28]. The current work is also consistent with the results obtained by other authors who reported allergenicity of α , β and γ gliadins in atopic dermatitis, and LMW glutenins in baker's asthma [20, 29, 30]. The study of Maruyama et al. [31] determined their relative reactivity in the following order: glutenins > gliadins > NGP. However, in the present study, the specificity of the obtained IgE-binding profiles was observed at the level of individual subunits and fractions of GP and NGP, which allowed to consider patterns of IgE binding as fingerprints characteristic for individual patients. This variation confirmed our hypothesis that the immunological response to wheat proteins is highly specific. The identified protein bands showed very strong, very weak or a complete absence of luminescence signal depending on the wheat variety and serum applied. This indicated that the combination of immunoreactive bands was associated with both patient and wheat genotype.

Most of the research on wheat allergenic proteins has thus far concerned ω -5 gliadins. It seems obvious as the ω -5 gliadin fractions act as the main allergens in WDEIA [18, 32–34]. However, ω -5 can be also active in other wheat allergies [21, 35]. Hence, we could expect the detection of ω -5 by at least some of the sera tested. The study showed that only one patient's (3) IgEs bound 60-kDa ω -5 gliadin derived from the 3xC wheat, while no immunodetection was observed in 3xN wheat samples. This finding suggests that patient 3 may be potentially threatened by WDEIA, while consuming food products containing flour from the 3xC type wheat. This risk, however, may be theoretically eliminated by substituting the latter for the 3xN wheat of potentially hypoallergenic properties in WDEIA.

Despite the high specificity of the IgE binding profiles, certain immunodetected proteins are likely important allergens, common for all patients examined. Taking into consideration the intensity of chemiluminescence signals and

the frequency of immunodetection by individual sera, the highest immunoreactivity was observed for 43-kDa and 47-kDa LMW glutenin subunits and 40-kDa γ -gliadin fraction. The observed immunodetection of 49-kDa NGP is especially worth to emphasize as it has never been reported as wheat allergenic protein before. Other studies frequently reported NGP proteins of lower MW 12–14 kDa as highly allergenic proteins [23, 24, 36]. In this study NGP of higher molecular weight (between 20 kDa and 65 kDa) were analyzed. All three patients, whose IgE sera detected the 49-kDa NGP have been also diagnosed with asthma symptoms, which suggests this protein may also plays an important role in disease development. Identification of the most allergenic components among GP and NGP is necessary for further development of hypoallergenic wheat genotypes lacking harmful proteins, tailored to the specific immunological response of individual patients.

The obtained results showed – to some degree – weakness of the present diagnostic methods that exclusively signalize whether examined patients are gluten sensitized or not. As demonstrated in this paper allergic reaction of individual patients may be different and strongly depends on combination of subunits and fractions of gluten and nongluten proteins. Based on the immunoblots it is possible to elaborate methodology of patient's sensitizing level prediction what probably relates to number and intensity of immunodetected protein bands. Hence, our results make basement for improving diagnostic methods used in allergology.

Comparison of immunoreactivity of native and reduced proteins allow to hypothesize whether conformational or linear epitopes play more important role in the IgE binding [30, 31]. Our findings evidently demonstrated no IgE detection of non-reduced gliadins, which indicated that α , β and γ fractions gained immunoreactivity only after the disruption of SS bonds and unfolding of protein molecules. This in turn may suggest an important role of linear epitopes in the IgE binding. Potentially, a more extended structure of the molecule after the reduction of disulphides facilitate recognition of linear epitopes by IgE antibodies.

CONCLUSIONS

- 1) Sensitizing properties of wheat are due to particular components among gluten protein and on-gluten proteins, rather than the whole gluten complex.
- 2) Immunoblotting patterns of allergenic wheat kernel proteins are highly specific with regard to both patients and wheat genotypes.
- 3) The most immunoreactive wheat proteins are the 43 kDa and 47 kDa subunits of the low-molecular weight glutenins, as well as 40 kDa γ -gliadin fraction and the 49 kDa fraction of non-gluten proteins.

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