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FLOUR QUALITY AND BINDING OF IMMUNOGLOBULIN E BY GLIADIN PROTEINS OF TWO WINTER WHEAT GENOTYPES*

Jacek Waga[1], Jerzy Zientarski[1], Krystyna Obtułowicz[2], Barbara Bilo[2]

[1]Department of Cereals Breeding and Quality Evaluation, Plant Breeding and Acclimatization Institute, Cracow;
[2]Department of Clinical and Environmental Allergology, Collegium Medicum, Jagiellonian University, Cracow

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Gliadin proteins of two winter wheat genotypes (the cultivar Ostka strzelecka and the line 60A) were analysed by ELISA using serum with a high content of IgE antibodies to gluten, obtained from patients showing symptoms of food and skin allergies. These wheats differed strongly in gliadin composition and gluten rheological properties. Two extraction methods were employed: a single-step method using 70% ethanol and a two-step method using 0.01 mol/L NaCl followed by 70% ethanol. ELISA results for Ostka strzelecka were much higher than those for line 60A at high antigen concentrations (dilutions 1:10 and 1:100); at lower antigen concentrations (dilutions 1:10.000 and 1:100.000), however, the opposite was observed. We also found that proteins extracted using the two-step method bound IgE antibodies much more strongly than those from the single-step extraction. This suggests that gliadin epitopes may be blocked by albumins and globulins that weaken the binding of IgE antibodies.

INTRODUCTION

Prolamins, albumins and globulins are the most important protein groups of wheat grain endosperm. Prolamins include a number of gliadin and glutenin fractions which are storage proteins [Bietz, 1979; Galili & Feldman, 1983]. Their specific chemical primary and secondary structures largely influence the unique viscoelastic properties of the gluten network [Kączkowski, 2002, Tatham *et al.*, 1990]. Albumins and globulins, in contrast, are mainly enzymatic proteins [Pogna *et al*., 1991]. They include trypsin and α-amylase inhibitors which help to protect mature grains against insects and fungal pathogens [Breitender & Ebner, 2000]. Both storage and enzymatic proteins are strong allergens, able to evoke gastrointestinal, respiratory and skin allergic symptoms in hypersensitized persons. However, the strongest allergenic properties are demonstrated by gliadins and albumins [Sandiford *et al.,* 1997; Armentia *et al*., 2002; Morita *et al*., 2003].

Gliadins are highly polymorphic proteins. Differences in their physicochemical structures are determined by genetic factors. Complex loci that code gliadins (the so-called "gene clusters") are located on the short arms of six chromosomes, *1A, 1B, 1D, 6A, 6B* and *6D* [Payne, 1987]. The manner in which they are inherited makes it possible to develop new genotypes containing various combinations of proteins which may relate to quality factors [Waga & Węgrzyn, 2000; Wesley *et al*., 1999]. In plant breeding, favourable protein variants from different genotypes are commonly used to improve the technological properties of new wheat cultivars [Bushuk, 1998; Payne *et al*., 1984].

Variability of protein structure probably also affects the efficiency of binding of different gliadin fractions by IgE antigliadin antibodies. A higher amount of these antibodies in serum of allergic persons is a typical symptom in food allergies [Battais *et al*., 2003; Lehto *et al*., 2003; Morita *et al*., 2001]. Differences in gliadin allergenicity among varieties are likely to enable the breeding of wheats with improved quality and health characteristics.

Contrary to gliadins, albumins and globulins are not highly polymorphic proteins (*i.e*. there are not complex loci with multiple alleles) and, in spite of their heterogeneity, have similar chemical properties and reactivities [Poerio *et al*., 1991]. However, albumins and globulins can be bound by IgE antigliadin antibodies from serum of patients with allergy symptoms, suggesting the presence of similar epitopes in albumins, globulins and gliadins [Sandiford *et al*., 1997; Simonato *et al*., 2001]. Their interactions can modify the immunological response in sensitized persons.

The allergenicity of wheat grain proteins has been intensively studied. In gluten proteins research, the method of isolation is a very important factor influencing objectivity of results [Shewry, 2003]. Ethyl alcohol (mainly 70% solution) is commonly used to extract gliadins from wheat flour for analysis [Bietz, 1979; Wieser, 1991; Weegels *et al*., 1995]. Resulting extracts, however, contain albumins and globulins (soluble in water and aqueous saline solutions according to Osborne & Harris [1906]) as well as gliadins. Thus, immunological reactions between extracted proteins and patients' serum IgE antibodies to gluten may reflect the allergenicity of all three protein fractions. Characteristics of the immunogenic activity of pure gliadin proteins requires no presence of albumins and globulins. A comparison of the binding ability of IgE antibodies to purified and partly purified gliadins should reveal whether and how albumins and globulins may modify gliadin allergenicity.

Author's address for correspondence: Dr. Jacek Waga, Department of Cereals Breeding and Quality Evaluation, Plant Breeding and Acclimatization Institute, ul. Zawiła 4, 30-423 Kraków, Poland; tel.: (48 12) 266 57 00, fax. (48 12) 266 59 48, e-mail: zhwaga@cyf-kr.edu.pl

The objective of this research was to determine whether chosen winter wheat genotypes, that strongly differ in flour quality characteristics and gliadin composition, differ also in respect of binding ability of serum IgE antibodies to gluten with gliadin proteins, and how the method of protein extraction influences the effect of their immunological reaction.

MATERIAL AND METHODS

Extraction of gliadins. Two winter wheats, cultivar Ostka strzelecka and line 60A, harvested in 2004, were selected from the collection of the Plant Breeding and Acclimatization Institute in Cracow (PBAI) for analysis. About 1 kg of grain from each wheat was milled in a Quadrumat Senior (Brabender GmbH, Germany) laboratory mill.

Gliadins were extracted from flour by Osborne & Harris [1906] stepwise extraction modified by us as a single-step and a two-step method. In the single-step method, 5 mL of 70% ethyl alcohol were added to 0.5 g of flour; the mixture was gently shaken overnight, and then centrifuged for 15 min at 10,000 rpm in an Eppendorf 5810 centrifuge. The supernatant was saved for analyses. In the two-step method, albumins and globulins were first extracted for two hours at room temperature with 0.01 mol/L NaCl (1:10 w/v); the slurry was then centrifuged and the supernatant discarded. Subsequently, 5 mL of 70% ethanol were added to each centrifuge tube, and the contents were gently shaken overnight and centrifuged. The resulting supernatant was saved for further analyses.

A-PAGE electrophoresis. Wheat gliadin polymorphism was determined by acidic polyacrylamide gel electrophoresis in lactate aluminium buffer (pH=3.1) [Bushuk & Zillman, 1978]. Before analysis, gliadin extracts were concentrated with saturated sucrose solution in lactate aluminium buffer, and 10 μL portions were added to wells in the gel plate. Buffer chambers were filled with lactate aluminium buffer. Proteins were separated in a gel with total monomer concentration of T=8% (w/v) and crosslinker (methylene bisacrylamide) concentration of C=0.29% (w/v) at a constant voltage of U=500 V for *ca*. 3 h using a Desaphor apparatus (Desaga GmbH, Germany). After analysis, the gels were stained overnight in a Coomassie Brilliant Blue (R 250 + G 250) solution with added methanol (17%) and acetic acid (5%) and destained in distilled water for *ca*. 24 h. Electrophoretic patterns were interpreted using the catalogue of gliadin protein blocks worked out at PBAI [Waga, 2000].

Technological properties. The total protein content was determined by near infrared reflectance (NIR) using the Tecator Infratec 1255 analyser. The sedimentation value was determined by the Zeleny method as modified by Greenway on a micro scale, using 320-mg flour samples with 2% sodium dodecyl sulfate added [Zeleny *et al*., 1960].

Rheological properties of wheat flours were characterised with a 50-g farinograph (Brabender GmbH, Germany). Analyses were carried out in duplicate using the standard method ISO 5530-1 [1988]. Results obtained were water absorption value, dough development time, dough stability time, degree of softening after 12 min and quality number.

Dough extensibility was analysed using a 300-g extensograph (Brabender GmbH, Germany) using the standard method ICC 114/1 [1992]. Based on the extensograph analysis three indicators were determined. Analyses indicated dough energy, dough resistance and dough extensibility.

ELISA. Binding of serum IgE antibodies to gluten with gliadin proteins was determined by indirect enzyme-linked immunosorbent assay (ELISA) based on the modified method described by Battais *et al.* [2003]. Six successive gliadin dilutions in carbonate buffer, pH=9.6, ranging from 1:10 to 1:1.000.000, were analysed. Microtitration plates were coated with diluted gliadin extracts (100 μL per well). Each dilution was analysed in four replications. The plates were incubated overnight at 4°C, and then blocked for two hours with 1% fetal calf serum solution in a buffered sodium chloride solution with the addition of 0.05% Tween (PBST-FCS) at room temperature. After removing the blocker, 100 μL of human serum, diluted in PBST(1:10), were added to each well.

These sera were obtained from eight patients with wheat allergy, treated at the Department of Clinical and Environmental Allergology, Collegium Medicum of the Jagiellonian University, who showed a high level of IgE antibodies to gluten. The collected sera samples were pooled and stored at -20°C.

Serum was incubated for two hours at 37°C. Next, the conjugate solution (IgE antihuman antibodies coupled with horseradish peroxidase, Sigma Chemical Co., USA) diluted at 1:1000, was added to each well and incubated for two hours at 37°C. After each step of the analysis, wells were rinsed three times with PBST solution. Orthophenylenediamine (Sigma Chemical Co., USA) was used as a substrate for detecting the activity of the marking enzyme. Substrate solution was prepared from tablets before use according to manufacturer's instructions. The reaction was carried out in the dark for 20 min, and then stopped with 1 N H_2SO_4.

The indicator of binding ability of serum IgE to gliadin proteins was the value of optical density (OD) of the coloured immunoenzymatic reaction product, read by an Opsys MR ELISA test reader (Thermo Lab System, USA) at 490 nm.

RESULTS AND DISCUSSION

Electrophoresis showed that Ostka strzelecka and line 60A differed considerably in gliadin composition (Figure 1). Interpretation of electrophoretic patterns was based on the concept of protein blocks resulting from the theoretical principles of wheat storage protein inheritance. Each of the six gliadin-coding loci contains several closely linked genes. Each locus contains one of several alleles, each of which codes 1–6 proteins. Upon electrophoresis, the resulting groups of bands are referred to as protein blocks [Metakovsky, 1991]. The analysed forms of winter wheat genotypes differed in five of the six block patterns typical of hexaploid wheat. Fractions coded by chromosome *6D* in Ostka strzelecka were especially interesting and unique; as this chromosome appears to code no proteins, and to have a null allele. So far the null allele has not been observed in gliadins of Polish wheat cultivars and breeding strains.

Technological analyses showed considerable differences between the two genotypes analysed (Table 1). Quality parameters of 60A were typical of medium or low-quality

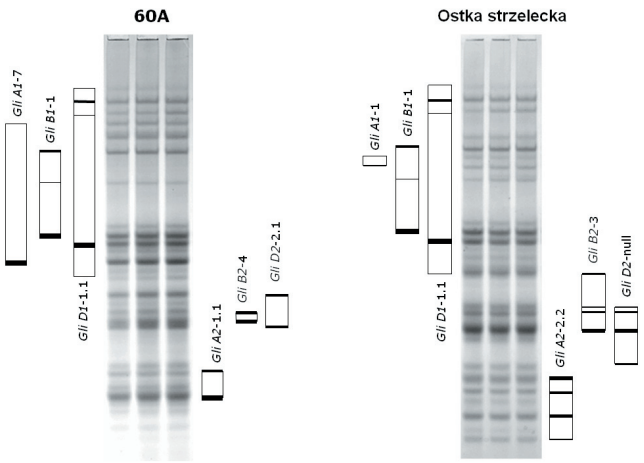

FIGURE 1. A-PAGE paterns and schemes of gliadin protein block for two winter genotypes: the line 60A and the cultivar Ostka strzelecka.

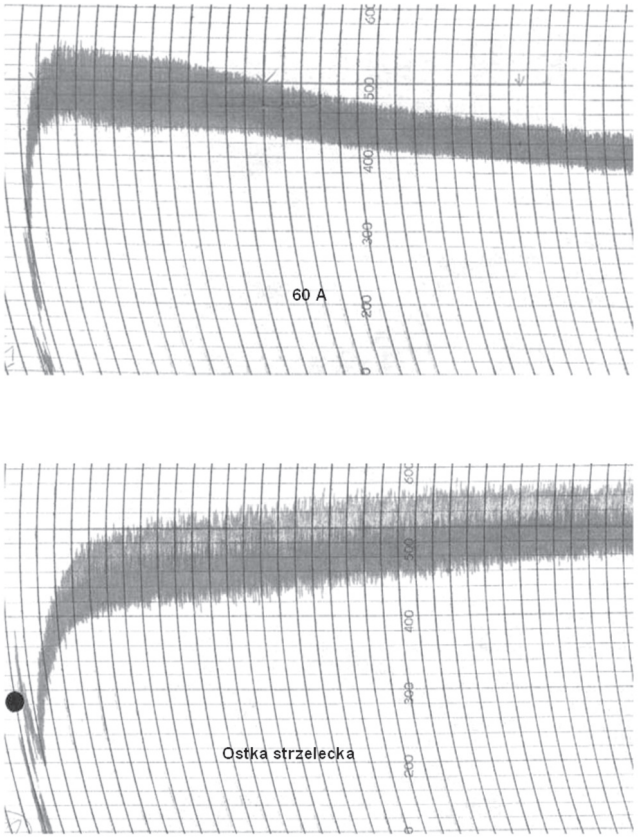

FIGURE 2. Comparision of farinographic plots of two winter genotypes: the line 60A and the cultivar Ostka strzelecka.

wheat, while those of Ostka strzelecka were uniquely favourable. Total protein content and sedimentation value indicated that Ostka strzelecka had proteins of superior quality to 60A. This was also confirmed by farinographic and extensographic analyses. Values of all parameters from farinographic analyses were more favourable for Ostka strzelecka. Degree of softening for this variety, which has a minimal value, is especially noteworthy: dough made from this flour exhibited no softening whatsoever (Figure 2). Only a fragment of the Ostka strzelecka farinographic curve was shown; after achieving the 500 BU line, the dough remained stable for a prolonged period. Once the time of analysis was increased even by a factor of three, no decrease below the 500 BU dough stability limit occurred. Thus, Ostka strzelecka formed a very strong gluten. This was confirmed by an extensographic analysis: the area under the extensograph curves and the shape of their lines indicated that the energy, resistance and extensibility of dough were much greater for Ostka strzelecka than for the line 60A (Figure 3). Thus, technological tests showed significant differences between the analysed genotypes in the physicochemical properties.

The binding ability of serum IgE antibodies to gluten with gliadin proteins analysed with ELISA was different when comparing both genotypes. Higher OD values for Ostka strzelecka at higher antigen concentrations (dilutions ranging from 1:10 to 1:1000) than for line 60A were observed (Figure 4). At a 1:1000 dilution, binding of IgE by gliadins was almost equal for both wheats, whereas at low gliadin concentrations (1:10.000 and 1:100.000), an inverse relation was observed: OD values for 60A were higher that for Ostka strzelecka. These relationships were found for gliadins extracted both with the single-step and two-step extraction methods.

Considerably greater differences in the strength of IgE antibody response to gliadin were found when comparing ELISA results for the two antigen extraction methods (Figure 5). Surprisingly, OD values were much higher for the two-step than the single-step extract. The difference was especially great (almost 5x) at a gliadin dilution of 1:10. When gliadin concentration decreased, OD value difference decreased as well. At dilutions of 1:10.000, 1:100.000 and 1:1.000.000, immunoenzymatic reaction results were similar for both extraction methods.

The present studies of IgE binding to gliadin proteins have yielded surprising results, difficult to compare with results of other authors [Battais *et al*., 2003; Lehto *et al*., 2003; Sandiford *et al*., 1997]. The immunoenzymatic reaction, as estimated by ELISA, indicated that wheat gliadins bind IgE antigliadin antibodies in human serum in different ways, depending on antigen concentration. When antigen concentration was high (1:10 and 1:100 dilutions), binding of IgE by gliadins was stronger in Ostka strzelecka than in 60A. An opposite relationship was found at low concentrations (1:10.000 and

TABLE 1. Technological characteristics of two winter wheat flours used in this study.

Wheat genotype	Sv	P	Fy	Farinograph					Extensograph		
				Wa	Ddt	Dst	Qn	Ds.	Den	Dr	Dex
60A	27	10.7	76.0	59.4	1.8	6.0	70	80	63	250	147
Ostka strzelecka	67	17.8	71.8	64.1	13.0	20.0	200	1	170	371	206

Sv – sedimentation value (mL); P – total protein content (%); Fy – flour yield (%)
Farinograph parameters: Wa – farinograph water absorption value (cm^3/100 g); Ddt – dough development time (min);
Dst – dough stability time (min); Qn – quality number (mm); Ds – degree of softening after 12 min (BU)
Extensograph parameters: Den – dough energy (cm^2); Dr – dough resistance (BU); Dex – dough extensibility (mm)

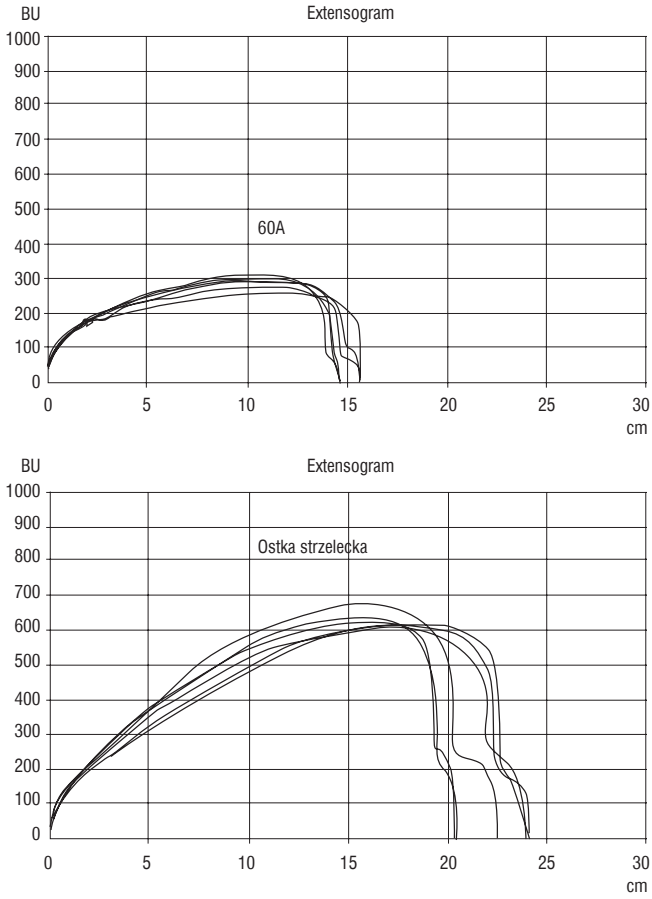

FIGURE 3. Comparision of extensographic plots of two winter genotypes: the line 60A and the cultivar Ostka strzelecka.

1:100.000 dilutions). Higher OD values for wheat of a higher total protein content at a high antigen concentration suggest that under such conditions IgE binding ability is determined to a greater extent by protein quantity than by such quality features as structural characteristics. However, the opposite relationship at low antigen concentrations suggests a greater influence of structural traits on the binding of immunoglobulin E. Thus, it appears that IgE binding to gliadin proteins results both from protein content and structural traits.

Results from the comparison of gliadin extraction methods are more controversial. The stronger binding of IgE antibodies by proteins extracted using the two-step as compared with the single-step method appears opposite to theoretically expected results. With two-step extraction, the extract contains only one group of proteins, gliadins. With one-step extraction, albumins and globulins, as well as gliadins, are present. Results of other authors show that water- and salt-soluble proteins can react with IgE antigliadin antibodies produced in serum of allergic persons [Sandiford *et al*., 1997; Mills *et al*., 2002]. Moreover, the presence of anti-albumin and anti-globulin antibodies in the analysed sera cannot be excluded [Garcia-Casado *et al*., 1994]. Thus, it might be expected that the mixture of three antigens would react more strongly with antibodies in human serum than a single antigen. However, the opposite was observed. In our opinion, albumins and globulins in a solution weaken the immunoenzymatic reaction of gliadins, suggesting that, for patients studied, gliadins are a stronger allergen than albumins and globulins. Another study, however, suggests that albumins can be more allergenic than gliadins [Ikezawa *et al*., 1994]. Our results

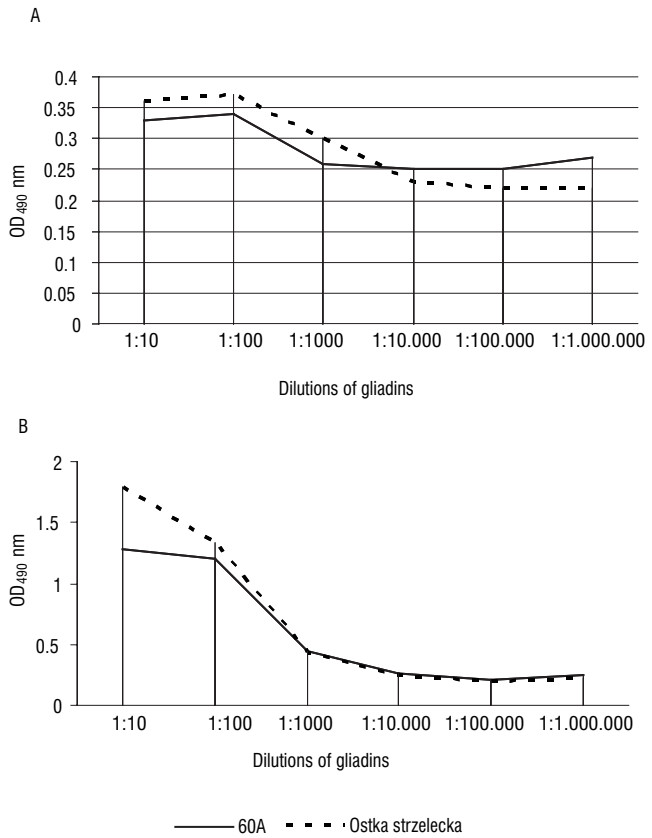

FIGURE 4. Influence of genotype on the OD value for two gliadin extraction methods used in this study: A – one-step extraction, B – two-step extraction.

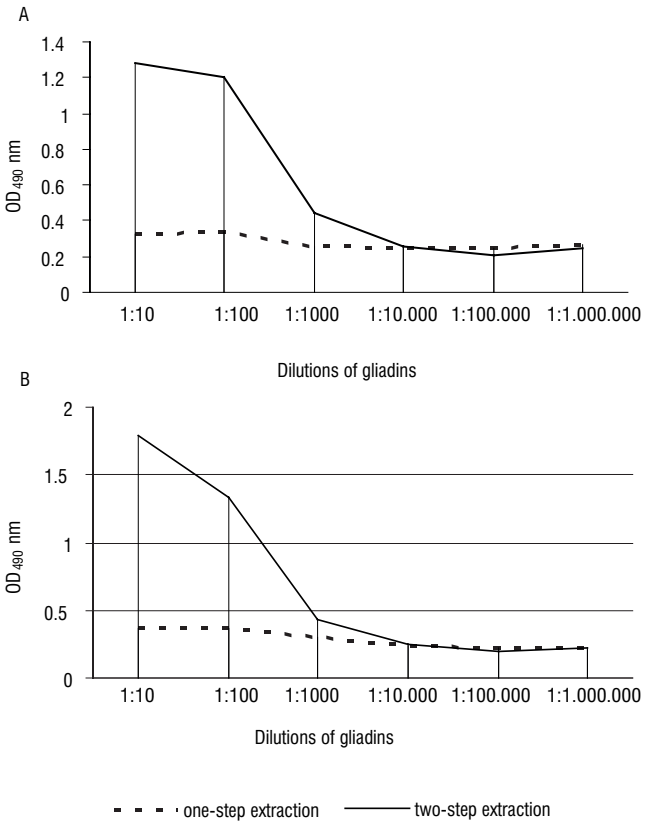

FIGURE 5. Influence of the method of gliadin extraction on the OD value for analysed winter wheat genotypes: A – the line 60A, B – the cultivar Ostka strzelecka.

may also indicate that stronger binding of IgE antibodies by purified gliadins – as compared with the protein mixture – is due to the interaction at the primary and secondary structure level that may block IgE binding epitopes of gliadins. Various interactions among wheat endosperm proteins have been demonstrated [Bushuk, 1993]. After removing albumins and globulins, which interfere with immunological reaction, antibodies regain full access to binding epitopes, as reflected by spectrophotometry. This hypothesis is partly confirmed by later results (unpublished) in which we compared the immunoreactive properties of gliadins extracted using the single- and two-step methods by the "sandwich ELISA" using rabbit polyclonal antigliadin antibodies. Although initially extracting albumins and globulins with 0.01 mol/L NaCl did not increase gliadin immunoreactivity, a more concentrated extracting agent (0.1 mol/L NaCl) in the first step gave an effect analogous to that described in this paper.

Both hypotheses concerning blocking of gliadin structural epitopes by albumins and globulins and the relationships between the antigen content, structure and allergenicity need to be verified by clinical research. For this reason, the differences in the binding ability of serum IgE antibodies to wheat gliadin proteins and flour technological properties shown in this study are important. Confirmation in further experiments may make it possible to improve the health and quality characteristics of wheat cultivars using plant genetics and breeding methods.

CONCLUSIONS

1. Wheat genotypes that differ greatly in the composition of gliadins and technological properties of flour differ also in the binding of serum IgE antibodies to gluten with gliadin proteins.

2. Changes of antigen concentration alter the binding ability of serum IgE. At higher gliadin concentrations, probably it mostly depends on total flour protein content; at low concentrations, differences in the protein structure may be more important.

3. Albumins and globulins can alter IgE binding to gliadins by blocking structural epitopes, resulting in decreased human IgE antibodies to gluten immunological response.

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WŁAŚCIWOŚCI TECHNOLOGICZNE ORAZ CHARAKTERYSTYKA WIĄZANIA IMMUNOGLOBULINY E PRZEZ BIAŁKA GLIADYNOWE DWÓCH FORM PSZENICY ZWYCZAJNEJ (*TRITICUM AESTIVUM* L.)

Jacek Waga[1], Jerzy Zientarski[1], Krystyna Obtułowicz[2], Barbara Bilo[2]

[1]Zakład Oceny Jakości i Metod Hodowli Zbóż, Instytut Hodowli i Aklimatyzacji Roślin, Kraków,
[2]Zakład Alergologii Klinicznej i Środowiskowej, Collegium Medicum UJ, Kraków

Właściwości alergogenne gliadyn dwóch form pszenicy ozimej (odmiany Ostka strzelecka oraz linii 60A) o różnym składzie frakcji białkowych oraz różnych właściwościach technologicznych mąki badano przy użyciu testu ELISA. W reakcji immunoenzymatycznej wykorzystano surowice o podwyższonej zawartości przeciwciał antygliadynowych w klasie IgE, pobrane od pacjentów z objawami alergii pokarmowej i skórnej. Zastosowano dwie metody ekstrakcji: jednostopniową 70% etanolem oraz dwustopniową – w pierwszej kolejności 0,01 mol/L roztworem NaCl, a następnie 70% etanolem. W wysokich stężeniach antygenu (rozcieńczenia 1:10 i 1:100) wynik testu ELISA dla Ostki strzeleckiej był wyższy niż dla linii 60A, w niższym zakresie stężeń (rozcieńczenia 1:10.000 i 1:100.000) wystąpiła odwrotna zależność (rys. 4). Obserwowano również znacznie silniejsze wiązanie przeciwciał IgE przez białka ekstrahowane metodą dwustopniową niż jednostopniową. Uzyskane wyniki sugerują, iż zróżnicowanie właściwości alergennych gliadyn może być efektem wypadkowym stężenia antygenu oraz jego cech strukturalnych. Stwierdzono również możliwość blokowania epitopów gliadynowych przez albuminy i globuliny. Prawdopodobnie ich obecność w roztworze wpływa hamująco na przebieg reakcji immunologicznej gliadyn z antygliadynowymi przeciwciałami IgE obecnymi w surowicy wybranych pacjentów.