

Influence of Glycation and Pepsin Hydrolysis on Immunoreactivity of Albumin/Globulin Fraction of Herbicide Resistant Wheat Line

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Abstract

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The aim of this study was to investigate the influence of non-enzymatic glycosylation on the immunogenic properties of soluble wheat proteins. Albumin/globulin fractions of herbicide resistant wheat line were non-enzymatically glycosylated using glucose for seven days at 37°C. The changes in their structures and immunoreactivity were then determined. The protein fractions were also hydrolysed with pepsin to determine the resistance to digestion. Albumin/globulin fractions before and after non-enzymatic glycosylation were analysed using *o*-phthaldialdehyde method and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The immunoreactivity of the protein fractions was determined using enzyme-linked immunosorbent assay methods, as well as affinity chromatography. The soluble wheat proteins showed smaller amounts of available α -amino groups after non-enzymatic glycosylation, and were stronger immunogens after glycation, but their antigenicity was not been affected significantly. However, pepsin hydrolysis of wheat proteins decreased their immunoreactivity.

Keywords: non-enzymatic glycosylation; glycation; pepsin hydrolysis; immunoreactivity

Wheat is a major source of plant proteins with a high nutritional value, extensively used in the daily diet. The protein content of most commercial wheat samples varies between 8 and 16%, depending on the variety and growing conditions. The amino acid composition of the total protein of wheat kernel is characterised by high amounts of glutamic acid (and glutamine) and proline and low concentrations of basic amino acids, particularly lysine (LÁSZTITY

1999). Wheat proteins are generally classified on the basis of their solubility into water/salt soluble albumins and globulins and water/salt insoluble gliadins and glutenins, which are the major grain proteins (SHEWRY 1995); among them are also proteins, which may induce various clinical symptoms belonging to food allergy reactions. Hypersensitivity to wheat is encountered in infants and adults. Wheat flour induces allergic reactions by

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ingestion and inhalation. Wheat-sensitive atopic patients typically produce IgE antibodies against soluble grain proteins, but only a few have been shown to have gluten specific IgE antibodies. IgE antibodies to a number of wheat flour components have been found in patients with baker's asthma, the strongest reactivity being observed with wheat albumins and globulins.

Immunoreactivity of proteins can be changed by different modification processes or by food technology. Industrial processes or various types of cooking, as well as long-term storage of foods, provoke interactions between proteins and non-protein components. One of these interactions is the non-enzymatic glycosylation of proteins, called glycation. Glycation of proteins is a spontaneous reaction between reducing carbohydrates and free amino groups (e.g. in amino acids or the ϵ -amino group of lysine in proteins, as well as the α -amino groups of terminal amino acids) leading to the formation of Schiff base. This intermediate is unstable and undergoes rearrangement to form stable Amadori products. Subsequently, the Amadori products degrade into dicarbonyl intermediates. These compounds can form cross-links, stable end products, called advanced glycation end products (AGEs) (MARTINS *et al.* 2001). During non-enzymatic glycosylation, a wide range of reaction products are formed with a significant importance for the nutritional value of foods. This value can be reduced by decreasing the digestibility or changing the immunoreactivity of proteins (MALEKI *et al.* 2000; CHUNG & CHAMPAGNE 2001), or by a possible formation of toxic and mutagenic compounds. However, the nutritional value of proteins can be improved by the formation of antioxidative and antimutagenic products (MARTINS *et al.* 2001). In addition, it is very important to define and characterise modified proteins for a precise and rapid detection in complex food matrices, especially when these proteins may elicit immune response.

In this study, we investigated the changes in the immunoreactivity of water/salt soluble wheat proteins after non-enzymatic glycosylation and pepsin hydrolysis. The investigations were undertaken to answer the following question, i.e. whether biological and immunological properties of wheat proteins change after binding glucose. These changes can occur during different technological processes. In addition, we examined the impact of pepsin hydrolysis on the immunoreactivity of wheat proteins.

MATERIALS AND METHODS

Materials

- Spring bread wheat *Triticum Aestivum* L. (CY-45Q) as parent wheat.
- Transformed wheat line of *Triticum aestivum* L. (T-128Q) as a transformed wheat line obtained from field trial experiments (2002) of the Cereal Research Non Profit Co, Szeged, Hungary. The transgenic wheat line contains bacterial-derived alien gene (*bar*) under the corn ubiquitin promoter, which possesses resistance to the agents of the glyphosate (phosphinotrichin) family (PAUK *et al.* 2001).

Preparation of albumin/globulin fractions (AGF). The samples (18 g) of ground wheat flour were extracted with 300 ml of 0.5 mol/l NaCl at 4°C for 1 h by mixing and centrifuging (5000 g, 20 min). The supernatant, containing albumin and globulin proteins, was dialysed at 4°C for 48 h against distilled water and then lyophilised.

Determination of amino acid composition. The analysis of amino acids was performed after hydrolysis with 6 mol/l HCl at 105°C for 18 h in nitrogen atmosphere employing an amino acid analyser (Beckman 6300).

Non-enzymatic glycosylation (glycation) of soluble wheat proteins. Glycation (G) of soluble wheat proteins was performed according to the method described by NESSAR and FURTH (1991). 200 mg of soluble wheat proteins were dissolved in 40 ml of 0.2 mol/l phosphate buffer (pH 7.4), followed by the addition of 400 mg of glucose. As antimicrobial agent, 0.1 ml of 4% sodium azide was used. Incubation was carried out at 37°C for 7 days. After incubation, the samples were dialysed against distilled water at 4°C to remove unbound glucose.

Kinetic analysis of wheat proteins hydrolysis with pepsin. The kinetic analysis of the samples was carried out at pH 2.0. The starting solution was prepared as follows: 75 mg of wheat proteins were dissolved in 25 ml of distilled water at pH 2.0 (obtained by the addition of 1 mol/l HCl) followed by the addition of 2 mg pepsin. The incubation was carried out at 37°C for 2 h. The hydrolysis was stopped by heating at 90°C for 5 minutes. The amino compounds in the supernatant were determined using the OPA method (FRISTER *et al.* 1986).

Determination of available amino groups (OPA method). The quantity of the available amino groups was determined by the OPA method (FRIS-

TER *et al.* 1986) with modification (PANASIUK *et al.* 1998). To the aliquot 0.125 ml of the examined sample, 5 ml of freshly prepared OPA reagent was added. OPA reagent consisted of 160 mg *o*-phthaldialdehyde (OPA), 4 ml methanol, 0.4 ml mercaptoethanol, and 0.1 mol/l sodium borate buffer (pH 9.5) made up to 200 ml. The absorbance was measured at 340 nm after 20 minutes. Blank was made identically using 0.125 ml of water. The calibration curve was obtained by using 0.25–2 mmol/l L-leucine as a standard.

Determination of Degree of Hydrolysis (DH). The total content of α -amine groups (h_{total}) was determined according to the method reported by HAJÓS *et al.* (1988). The solution of 10 ml of 6 mol/l HCl and 0.5 g of protein extract was put in a glass ampoule and was saturated with nitrogen. The ampoule was closed by heating. Hydrolysis was conducted at 105°C for 12 hours. The solution was filtered and neutralised with 6 mol/l NaOH, and subsequently transferred to a volumetric flask and adjusted with phosphate buffer (pH 8.2) to a final volume of 100 ml. The content of α -amine groups was determined using OPA method. The degree of hydrolysis (DH) was calculated from the following equation (ADLER-NISSEN 1979):

$$\text{DH} = h/h_{\text{total}} \times 100\%$$

where:

h – number of hydrolysed peptide bonds (mEq Leu – NH_2 /g of protein)

h_{total} – total number of peptide bonds in the protein (mEq Leu – NH_2 /g of protein)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE electrophoresis was carried out according to LAEMMLI (1970). The SDS-gel contained 4% polyacrylamide stacking gel and a resolving gel of 15% polyacrylamide. The gels were stained with Coomassie Brilliant Blue R-250 (Serva Seim Biochemica GmbH, Heidelberg, Germany) and Pro-QTM Emerland 300 Glycoprotein Gel Stain Kit (P-21855) (Pierce, Rockford, USA). Low molecular weight markers ranging from 6.5 to 66.0 kDa obtained from Sigma (St. Louis, USA) (M-3913) were used as standards.

Peptide electrophoresis. Electrophoresis was carried out according to SCHÄGGER *et al.* (1985) with modification. The separation of peptides was performed with 4% stacking gel, 10% transitory gel, and 16.5% resolution gel. Peptide and protein

samples were diluted in the buffer containing 2.5% β -mercaptoethanol and 2% SDS. Ultra Low Range Markers (Sigma) ranging from 26.6 to 3.5 kDa were used as standards. The gel was run in 0.1 mol/l Tris-tricine buffer containing 0.1% SDS, pH 8.6 (top buffer) and 0.2 mol/l Tris-Cl buffer, pH 8.9 (bottom buffer). The peptides were stained with 0.025% Coomassie Brilliant Blue in 10% acetic acid.

Production of polyclonal antibodies. White rabbits were immunised with 0.5 mg of AGFs or glycated AGFs in PBS (pH 7.4). The protein samples were emulsified with equivalent volume (0.6 ml) of Freund's complete adjuvant (Sigma) for the first immunisation. Next three immunisations were performed at 14-days intervals in the presence of Freund's incomplete adjuvant. All immunisation injections were applied subcutaneously. The production of antibodies and the increase in their titres were controlled by indirect enzyme-linked immunosorbent assay (ELISA) taking blood samples from the marginal vein 2–3 days prior to the subsequent scheduled immunisation. A week after the last immunisation, the rabbits were exsanguinated. Blood samples were incubated at 37°C for 1 h followed by centrifugation (2000 g for 20 min), then the sera were split into aliquots and frozen at –20°C before analyses.

IgG purification. IgG was purified by precipitation with ammonium sulphate from the sera according to PAGE and THORPE (2002).

Indirect ELISA method. The microtiter plate (Nunc[®]) was coated with 5 $\mu\text{g/l}$ of antigen in 50 mmol/l carbonate buffer solution, pH 9.6, in the amount of 200 μl per well, and was incubated for 1–18 h at 4°C. The plates were then rinsed two times with deionised water and the residual free binding sites were blocked with 250 μl of 1% BSA (Sigma) in 10 mmol/l phosphate buffered saline, pH 7.4 (PBS) at room temperature for 1 hour. Afterwards, the plates were washed 5 times with 10 mmol/l phosphate buffered saline, pH 7.4, containing 0.5% Tween-20 (PBS-T). This washing system was used after each analytical step. The plates were washed and incubated at 37°C for 1 h with 100 μl of 10-fold-diluted antibody added to each well. After the next washing, plates were incubated for 1 h at 37°C with 100 μl of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) followed by washing and the addition of ABTS (Moss, Inc., Pasadena, CA). After 20 min, absorbance was read at 405 nm on an automated

plate reader (Jupiter, UVM 340). To calculate the antibodies titre, the maximum dilution of antibody was used gave immunochemical interaction of the antibody with the antigen.

Competitive ELISA method. Microplates were coated with 5 µg/ml of antigen in 50 mmol/l carbonate buffer solution, pH 9.6, in the amount of 200 µl per well. The Microplates with the antigen were incubated at 4°C for 12–18 h, and then rinsed two times with deionised water. The sites of the microplates, which were not saturated by the antigen, were filled with 250 µl/well of 1% BSA (Sigma) in PBS, pH 7.4, at room temperature for 1 hour. After that, the plates were rinsed five times with PBS-T. This procedure was repeated after each step of this method. The wells were filled with a sample containing the antigen and the polyclonal rabbit antibodies obtained for the given antigen (50 µl of each solution of an adequate concentration per well) and were incubated at 37°C for 1 hours. After rinsing the microplates, 100 µl of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) was added to each well and incubated at 37°C for 1 h followed by washing and the addition of ABTS (Moss). After 20 min, absorbance was read at 405 nm on an automated plate reader (Jupiter, UVM 340).

Affinity chromatography. 150 mg of purified IgG in 4 ml 0.1 mol/l sodium bicarbonate (pH 8.0), containing 0.5 mol/l NaCl, were bound to the resin, activated CH-Sepharose 4B (Sigma), according to the Product Information Sheet (Sigma). The unbound ligand was removed with 0.1 mol/l sodium bicarbonate and the column was washed with PBS

(pH 7.4). 10 mg of AGFs or glycated AGFs were applied onto the column at room temperature for 2 hours. The unbound antigen was washed out with PBS (pH 7.4), then the bound antigen was removed with 0.1 mol/l glycine-HCl buffer (pH 2.8) at 4°C and dialysed against distilled water.

RESULTS AND DISCUSSION

From the glycation point of view, the contents of lysine, histidine, and arginine are very important because these amino acids take part in the reaction between carbohydrates and free amino groups of proteins (CAZACU-DAVIDESCU *et al.* 2005; HENLE 2005). The amino acid composition of water/salt-soluble wheat proteins of both lines are presented in Figure 1. Generally, the content of every amino acid in the extract of the transformed wheat line (T-128) was lower except for histidine and methionine. The lower content of lysine and arginine in the albumin/globulin extract of T-128 wheat line compared to that in the CY-45 wheat line may suggest that T-128 (genetically modified line) would be less susceptible to non-enzymatic glycosylation.

SDS-PAGE electrophoregram of crude protein wheat extracts (CY-45 and T-128) is shown in Figure 2. Both wheat lines are characterised by similar electrophoretic pattern. Only a few bands were found, which seem to be the main proteins in the extracts of both wheat lines. Their molecular weights were about 14.2, 36, and 66 kDa with another one having a molecular weight higher than the standards used. According to the literature

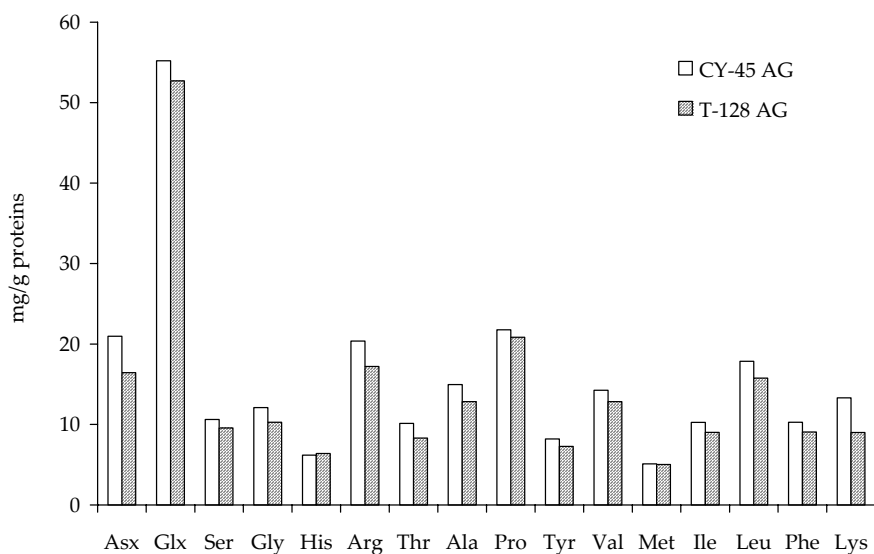
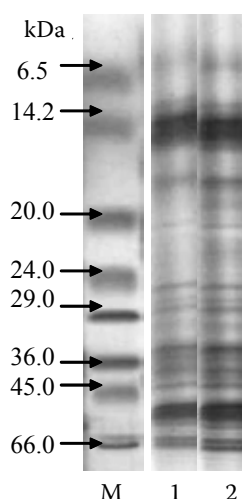


Figure 1. Amino acid composition of albumin/globulin fractions (AGF) of wheat lines



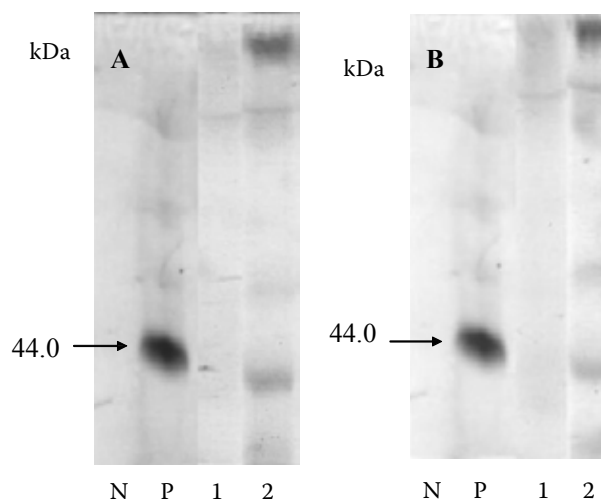
1 – CY-45 AGF; 2 – T-128 AGF; M – molecular weight marker

Figure 2. SDS-PAGE gel electrophoresis of wheat soluble proteins

(WEISS *et al.* 1997; MITTAG *et al.* 2004), those fractions represent the main immunoreactive proteins in albumin/globulin fractions.

In the next step of the experiment, those fractions by non-enzymatic glycosylation and pepsin hydrolysis to check, whether their immunogenic properties would change or not. The electrophoregram after SDS-PAGE separation of raw, autoglycated and glycated extracts was stained also for glycoproteins. Electrophoregram A and B in Figure 3 shows the differences between lines 1 (raw extract) and 2 (extract after glycation). Four fractions of glycoproteins could be noticed. These new glycoproteins could become a source of new epitopes, which may elicit different types of immunological response than the proteins before non-enzymatic glycosylation (CHUNG & CHAMPAGNE 2001).

The results of the replicate OPA method, which were obtained for the samples before and after glycation, are shown in Table 1. Although, the protein extract of T-128 can be characterised by a lower content of basic amino acids involved in glycation (Figure 1), no significant differences were observed between the amounts of available free amino groups. The content of available free amino acid groups decreased after glycation in both albumin/globulin wheat extracts, which also enhances sugar binding to proteins. The glycated protein extract of genetically modified wheat line (T-128) contained considerably fewer free amino



A – CY-45 (parent wheat); B – T-128 (genetically modified wheat); N – negative maker; P – positive control (horseradish peroxidase); 1 – native AGF; 2 – glycated AGF

Figure 3. SDS-PAGE gel electrophoresis of wheat soluble proteins stained for glycoproteins

groups than the protein extract of the parent wheat line (CY-45). According to this, it can be suggested that T-128 wheat line is more susceptible to glycation than the parent wheat line, which may be explained by different conformation of the parent wheat proteins that could hinder sugar binding to proteins. VAN DE LAGEMAAT *et al.* (2007) studied glycated soy proteins and also noticed a decrease in the content of available free amino groups after glycation, explaining this fact by protein aggregation and crosslinking.

The resistance to digestion is thought to be the crucial requirement for the allergenicity of food proteins. This resistance may be very important for both sensitisation of the immune system after penetration of the intestinal mucosa and for elicitation of gastrointestinal symptoms of food allergy (ASTWOOD *et al.* 1996; MOUÉCOUCOU *et al.* 2004). Hypersensitivity reactions after wheat

Table 1. The content of available free amino groups in native and glycated albumin/globulin fractions (AGF) after whole hydrolysis

	Free amino groups (μM Leu/mg proteins) ^a	
	native proteins	glycated proteins
CY-45 AGF	7.85 ± 0.18	7.57 ± 0.19
T-128 AGF	7.85 ± 0.22	5.29 ± 0.14

^aValues are mean \pm SD for each three replication used

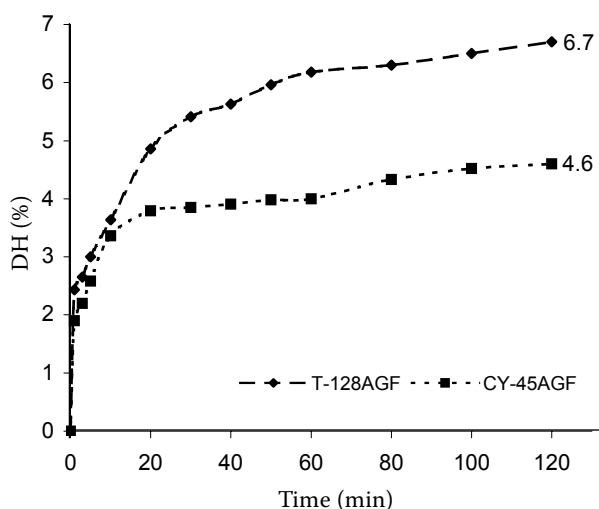


Figure 4. Kinetics of pepsin hydrolysis of wheat AGFs

ingestion have been reported by several authors (JAMES *et al.* 1997; MITTAG *et al.* 2004). Ingestion of foods containing wheat and other related cereals can induce gastrointestinal (GI) symptoms by either immunological or non-immunological mechanisms (SIMONATO *et al.*, 2001).

Figure 4 shows the kinetics of pepsin hydrolysis of both wheat protein extracts. The albumin/globulin extract of T-128 was more susceptible to

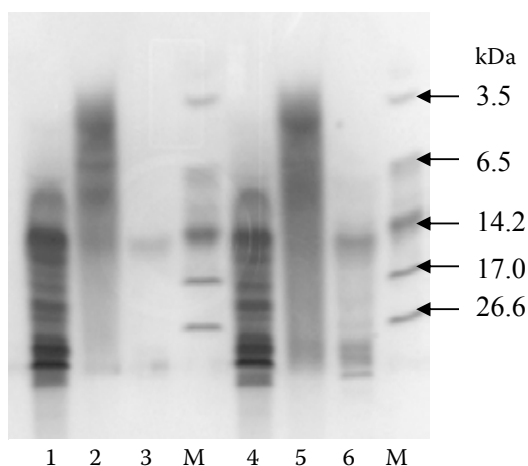


Figure 5. Peptide electrophoresis of wheat soluble proteins before and after affinity chromatography: M – molecular weight marker; 1 – CY-45 AGF; 2 – CY-45 AGF (after pepsin hydrolysis – H); 3 – protein fractions of CY-45 AGF (H) bound to polyclonal antibodies produced against CY-45 AGF using affinity chromatography; 4 – T-128 AGF; 5 – T-128 AGF (H); 6 – protein fractions of T-128 AGF (H) bound to polyclonal antibodies produced against T-128 AGF using affinity chromatography (AGF = albumin/globulin fraction; H = after pepsin hydrolysis)

the pepsin attack (DH 6.7%) in comparison with CY-45 (DH 4.7%). The resistance of CY-45 wheat line proteins to pepsin hydrolysis may indicate their higher immunoreactive properties. Peptide electrophoresis (Figure 5, lanes 2 and 5) shows the hydrolysates obtained from both wheat proteins extracts. After 2 h of protein hydrolysis, some non-hydrolysed proteins could still be noticed in both extracts although their amount was higher in the wheat parent proteins extract (CY-45).

The main aim of this study was to check the changes in the immunogenicity of wheat salt-soluble proteins caused by hydrolysis and glycation, therefore, rabbits were immunised with two crude extracts of wheat lines and their modifications (glycated and hydrolysates) to produce specific polyclonal IgG antibodies. The increase in the polyclonal antibodies titre after the immunisation of the rabbits was determined by indirect ELISA and the results are presented in Table 2. No differences could be observed between the serum titre against wheat proteins of CY-45 AGF and that of T-128 AGF, which means that both wheat lines may induce the immunological response in the organism with the same immune strength. Glycation increased the immunogenicity of proteins, resulting in about four times higher specific IgG titre as compared to that of the crude extract. Hydrolysis worked in the opposite way. The titre of specific sera IgG decreased about eight times in comparison with the crude extract. During hydrolysis, proteins are cleaved into smaller peptides that definitely affect their immunogenic properties (MILLS *et al.* 2003; WRÓBLEWSKA *et al.* 2004). Some of them are called haptens, in which case a special route of immunisation is needed to induce immunologic response (MCNAUGHT & WILKINSON 1997). The existence of peptide fractions was proved by peptide electrophoresis (Figure 5). It is clear that the mass distribution in hydrolysed samples changed Table 2. Antibodies titre against wheat albumin/globulin fractions before and after glycation and pepsin hydrolysis

Antigen	Antibody titer
CY-45 AGF	1:40 960
CY-45 AGF (G)	1:163 840
CY-45 AGF (H)	1:5 120
T-128 AGF	1:40 960
T-128 AGF (G)	1:163 840
T-128 AGF (H)	1:5 120

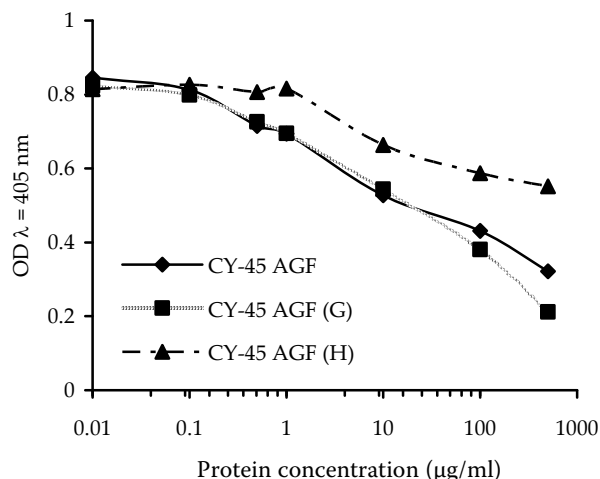


Figure 6. Cross reaction between wheat CY-45 AGF before and after glycation (G) and pepsin hydrolysis (H) using specific polyclonal antibodies against CY-45 AGF

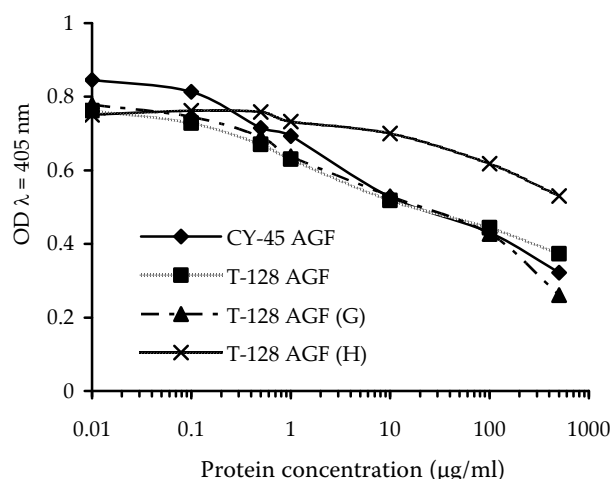


Figure 7. Cross reaction between wheat CY-45 AGF and wheat T-128 AGF before and after glycation (G) and pepsin hydrolysis (H) using specific polyclonal antibodies against CY-45 AGF

in comparison to crude extracts; new bands below 6.5 kDa were observed.

After immunisation, the specific rabbit sera obtained were used for specifying cross reactions. All combinations were checked (CY-45 AGF vs. CY-45 AGF (G), CY-45 AGF (H); CY-45 AGF vs. T-128 AGF, T-128 AGF (G), T-128 AGF (H); T-128 AGF vs. T-128 AGF (G), T-128 AGF (H); T-128 AGF vs. CY-45 AGF, CY-45 AGF (G), CY-45 AGF (H)). Figure 6 shows how antibodies produced against CY-45 AGF recognise glycosylated and hydrolysed wheat proteins. The curve for CY-45 AGF (G) is very similar to that for CY-45 AGF. It can be assumed that antibodies recognise different antigens in the same way. It means that the structure of epitopes did not change after glycation. The same

antibodies reacted much more weakly with the hydrolysed wheat proteins of CY-45 (Figure 6). It seems that pepsin hydrolysis lowered the immunoreactivity of wheat proteins.

Figure 7 presents the competition between two wheat species CY-45 AGF and T-128 AGF, T-128 AGF (G) and (H). The antibodies produced against CY-45 AGF recognised the proteins from T-128 AGF extract in almost the same way. Analysing the situation with the antibodies produced against T-128 AGF (Figure 8), it can be said that glycation did not significantly impact on the structure of the epitopes. The same cannot be stated about pepsin hydrolysis, where the hydrolysed wheat proteins were not recognised by the antibodies produced against native wheat proteins (T-128 AGF). The same applies to the antibodies produced against T-128 AGF which recognise the native and glycosylated wheat proteins of CY-45 line, but not the hydrolysed wheat proteins of that line (Figure 9). On the basis of the results obtained, it can be assumed that the immunoreactivities of proteins from both wheat lines are very similar despite the genetic modification of T-128. It may be suggested that the gene inserted into CY-45 does not change the sequence or the structure of the epitopes included in albumin and globulin wheat fractions; it only influences their biological properties. A similar situation occurred in the recognition of glycosylated proteins by antibodies produced against raw extract. However, there is only very weak or no competition between the

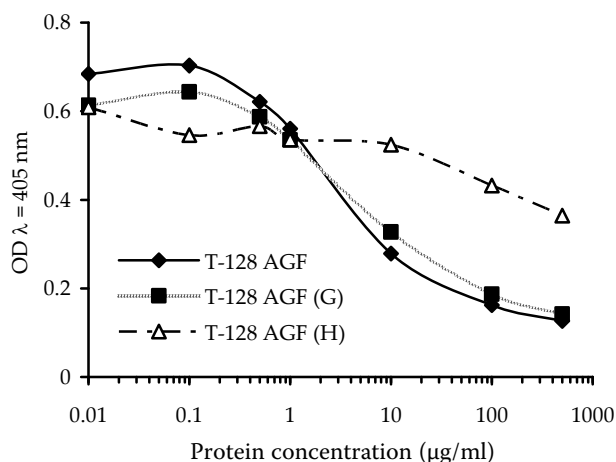


Figure 8. Cross reaction between wheat T-128 AGF before and after glycation

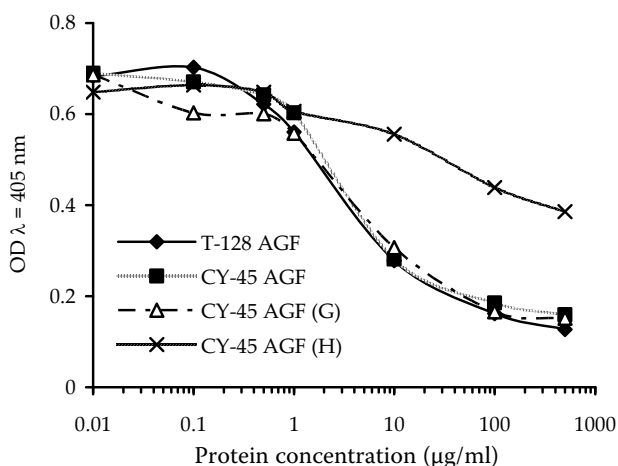


Figure 9. Cross reaction between wheat T-128 AGF and wheat CY-45 AGF before and after glycation (G) and pepsin hydrolysis (H) using specific polyclonal antibodies against T-128 AGF

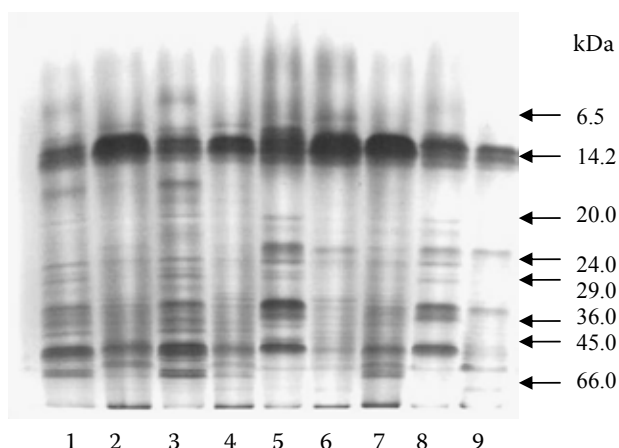


Figure 10. SDS-PAGE gel electrophoresis of wheat soluble proteins before and after affinity chromatography: M – molecular weight marker; 1 – CY-45 AGF; 2 – protein fractions of CY-45 AGF bound to polyclonal antibodies produced against CY-45 AGF after affinity chromatography; 3 – T-128 AGF; 4 – protein fractions of T-128 AGF bound to polyclonal antibodies produced against T-128 AGF after affinity chromatography; 5 – CY-45 AGF (G); 6 – protein fractions of CY-45 AGF (G) bound to polyclonal antibodies produced against CY-45 AGF (G) after affinity chromatography; 7 – protein fractions of CY-45 AGF bound to polyclonal antibodies produced against CY-45 AGF (G) after affinity chromatography; 8 – T-128 AGF (G); 9 – protein fractions of T-128 AGF (G) bound to polyclonal antibodies produced against T-128 AGF (G) after affinity chromatography (AGF = albumin/globulin fraction; H = after pepsin hydrolysis, G = after glycation)

antibodies produced against raw extracts of both lines and their hydrolysates (Figures 6–9), which proves a lower immunoreactivity of the hydrolysed proteins. Wróblewska *et al.* (2004) have also proven a reduction of immunoreactivity of whey proteins after enzymatic hydrolysis.

The produced specific polyclonal IgG were purified and used in affinity chromatography. This method was applied to determine the fractions mainly responsible for inducing the immunological response. Furthermore, the fractions were investigated for the changes caused by hydrolysis and glycation. As we can see in Figure 10, the antibodies produced against whole protein extracts recognised the main fractions with molecular weights of about 14.2, 24.0, 36.0, and 66.0 kDa. As mentioned above, these fractions were characterised as immunoreactive ones, according to the literature. We also determined the impact of glycation or pepsin hydrolysis on the immunoreactive properties of these proteins (Figures 10 and 11). In all cases, the main fraction recognised

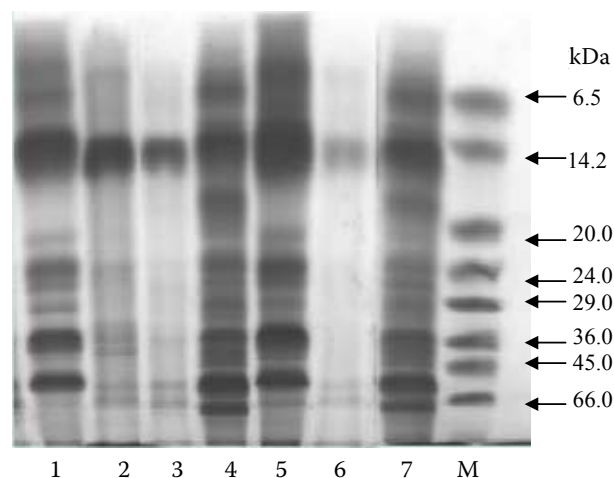


Figure 11. SDS-PAGE gel electrophoresis of wheat soluble proteins before and after affinity chromatography: M – molecular weight marker; 1 – T-128 AGF (G); 2 – protein fractions of T-128 AGF (G) bound to polyclonal antibodies produced against T-128 AGF after affinity chromatography; 3 – protein fractions of CY-45 AGF bound to polyclonal antibodies produced against T-128 AGF after affinity chromatography; 4 – T-128 AGF; 5 – CY-45 AGF (G); 6 – protein fractions of CY-45 AGF (G) bound to polyclonal antibodies produced against CY-45 AGF after affinity chromatography; 7 – CY-45 AGF (AGF = albumin/globulin fraction; H = after pepsin hydrolysis, G = after glycation)

by antibodies was the fraction with a molecular weight around 14.2 kDa (Figure 5).

CONCLUSIONS

According to the results obtained, it seems that in both wheat proteins extracts glycosylated products were successfully produced. In both cases, *in vitro* immunogenicity of these proteins increased after 7 days of non-enzymatic glycosylation (glycation), as measured by indirect ELISA, however using competitive ELISA the no significant changes in their immunoreactivity were observed. Pepsin hydrolysis significantly reduced the immunoreactivity of proteins in both wheat extracts.

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