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INFLUENCE OF GLYCATION OF WHEAT ALBUMINS AND GLOBULINS ON THEIR IMMUNOREACTIVITY AND PHYSICOCHEMICAL PROPERTIES

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Glycation (non-enzymatic glycosylation) is a spontaneous reaction that occurs during food processing, storage and preparation of food, which can have a significant influence on physiochemical and biological properties of food proteins. Glycation has been used mostly for improvement of functional properties of proteins although there is increasing concern of possible changes in biologic properties of glycation products.

The influence of dry-heat glycation with glucose on immunoreactive properties of wheat salt-soluble proteins has been studied. It has been shown that glycation caused differences in electrophoretic patterns (both 1D and 2D), as well as in Western-immunoblotting with rabbit IgG and human IgE. A significant increase in immunoreactivity has been observed, as well as a decrease in free amino group. Thermostable, immunoreactive and susceptible for glycation protein band with molecular weight of approximately 13 kDa (by SDS-PAGE) has been selected for the N-terminal sequence analysis and determined to be an alpha-amylase inhibitor.

Dry heat glycation is a very potent but nondestructive method of protein glycation, that leads to high degree of substitution and changes in both physicochemical and biological (immunological) properties of food proteins.

INTRODUCTION

Wheat is one of the most important crops in food and feed production as well as human nutrition – it holds the fourth place in the world crops cultivation, after sugar cane, maize and rice, with approximately 600 million metric tons of annual production (in year 2007, according to FAO – FAOSTAT [URL: http://faostat.fao.org/, access date 15.12.2008]). Wheat contains *ca.* 18% of protein with high nutritional value, although according to WHO standards it is not the protein of full value [Schaafsma, 2000]. Wheat proteins are "traditionally" classified according to their solubility as water-soluble albumins, salt-soluble globulins, alcohol-soluble gliadins and alkali-soluble gluteins.

An important issue concerning wheat is posed by allergenicity and toxicity of its proteins. Allergy to wheat (bakers' asthma) has been known since Ancient Rome, its symptoms were described in the early 18th century, while since the early 20th century it has been considered as an occupational disease [Jeffrey *et al.*, 1999]. Allergens for patients with atopic dermatitis with wheat allergy have been reported to be present in both saline-soluble and -insoluble fractions [Amano *et al.* 1998; Tanabe *et al.*, 1996] despite low resistance of the salt-soluble fraction to enzymatic hydrolysis [Mittag *et al.*, 2004], which is considered to be an important step in inducing allergic response [Taylor, 1997]. A genetic factor of celiac disease has been recognized as well [Moustakas *et al.*, 2000], still there are gliadin-derived peptides that take the responsibility.

Wheat is consumed in a processed form – *e.g.* as bakery products – and its food processing involves adding new ingredients as well as chemical, physical and thermal treatment. All of those actions may lead to changes in protein properties and structure [Davis *et al.*, 2001]. One of the most common chemical reactions in food processing is glycation (non-enzymatic glycosylation) of proteins with reducing sugars. Glycation is a two-step process – the first step is reversible formation of aldimine (Schiff base), the second is irreversible Amadori rearrangement that leads to the formation of more stable ketoamine, which also may be subject of further modifications. Sites of glycation are usually N-terminal amino groups of protein and amino groups of lysine [Tessier, 2010] and arginine [Lederer & Buehler, 1999]. Glycated arginine cross-links with lysine lead to the formation of derivatives with heterocyclic two-ring structure similar to diazepine [Ibid.].

In theory, glycation occurs in any situations where proteins and reducing sugars are present but it is most intense at basic pH, high temperatures and low water activity. To give few examples – it has been known since the last few decades that glycation occurs in physiologic conditions of living organisms [Tessier, 2009], during lyophilization [Zheng *et al.*, 2006], drying [Oliver *et al.*, 2006] and even cooking [Foerster & Henle, 2003]. Glycation has been used mostly for improvement of functional properties of proteins [Chevalier *et al.*, 2001; Li *et al.*, 2005; Achouri *et al.*, 2006] although there

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is increasing concern of possible changes in biologic properties of glycation products [Davis *et al.*, 2001; Lagemaat *et al.*, 2007; Wal, 2003; Mills *et al.*, 2006].

In case of wheat, glycation may occur during storage of seeds [Murthy & Sun, 2000], flour and its final product or during thermal processing like drying, extrusion, frying or baking. Glycation in wheat may occur by the so-called "autoglycation" with free soluble sugars or with sugars added during processing.

Among various thermal treatments and conditions, dry-heating (50-70°C) seems to be the most preferable for investigating glycation of food proteins for several reasons – at that temperature the reaction is approx. 7 times faster than at the physiologic temperature of 37°C (assuming that glycation as non-enzymatic reaction is subject of van't Hoff principle). Waterless conditions facilitate the first step of the glycation reaction (which normally may be reversed by the presence of water). Also, in this temperature range protein unfolds, exposing moieties which are normally hidden but the primary structure is not disrupted.

The aim of this study was to determine the influence of non-enzymatic glycosylation (glycation) by dry heating on physiochemical properties and immunoreactivity of salt-soluble wheat proteins.

MATERIAL AND METHODS

Protein extraction

Wheat grains (winter variety *Sukces*) were ground to fine powder with an electric laboratory mill. Proteins were extracted from flour with 0.15 mol/L NaCl (1:4 w/v) and shaken for one hour at 4°C [Sanchez-Monge *et al.*, 1986]. Then the extract was centrifuged for one hour at 15,000×g at 4°C and supernatant was collected. Three phase partitioning (TPP) [Dennison & Lovrien, 1997] was used for coarse purification and concentration of the sample – *t*-butanol was added to the protein extract to reach a concentration of 20%, followed by the slow addition of dry ammonium sulphate in the amount required to reach 100% saturation. After one hour of stirring at room temperature, the solution was centrifuged at 15,000×g for 15 min. The protein precipitated as a layer between *t*-butanol and saturated ammonium sulphate was collected, dissolved in phosphate buffered saline (PBS), dialyzed against the same buffer for 24 h and lyophilized. The protein content in the preparation was determined with BCA assay according to manufacturer's instructions (kit from Pierce).

Protein glycation

Glycation of the proteins by dry heating was conducted as follows – 50 mg of the protein and 50 mg of glucose were dissolved in 5 mL of PBS (control sample contained no glucose), frozen, lyophilized and subjected to temperature of 60°C for three days in sealed vials. After glycation, all samples were dissolved in 5 mL of water and dialyzed against PBS for 24 h.

Electrophoresis

The samples were separated by using a SDS-PAGE according to Laemmli, with 4% stacking gel and 15% resolving gel, under reducing conditions and voltage stabilized at 120 V. The gels were stained with colloidal Coomassie Brilliant Blue R-250.

Two-dimensional electrophoresis

The desalted and freeze-dried samples were dissolved in sample buffer (containing 8 mol/L urea, 2 mol/L tiourea, 2% CHAPS, 50 mmol/L DTT), applied on 7 cm pH 3-10 ReadyStrip IPG and run on PROTEAN IEF Cell for approx. 12 h. After the run, the gel strip was placed on top of SDS-PAGE gel and electrophoresis was run as described above.

Production of polyclonal antibodies

To prepare a single dose of vaccine 1 mg of protein was dissolved in 1 mL of sterile PBS and emulsified with equal volume of Freund adjuvant. Complete Freund adjuvant (CFA) was used in the first dose and incomplete Freund adjuvant (IFA) in the following one. Rabbits were immunized in one-week intervals and bled 10 days after the sixth dose. Antibodies were obtained from rabbits serum by ammonium sulphate precipitation (at 50% of saturation), dialysed against PBS (24 h at 4°C), lyophilized and used for quantitative ELISA and Western-immunoblotting.

Western blotting

Proteins were transferred from SDS-PAGE gels to PVDF membrane (Immobilon-P^{SQ}, 0.22 μm, Millipore) using Towbin buffer in semi-dry conditions (Trans-blot SD, BioRad) for 60 min at 0.8 mA × cm^2. After an overnight blocking with Tris Buffered Saline with 0.1% Tween-20 (TBS-T) the blot was probed with antibodies – rabbit polyclonal antibodies (0.1 mg/mL in TBS-T) for 60 min and washed 3 times in TBS-T. The blot was incubated for one hour with anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase (dilution 1:10000 in TBS-T), washed 3 times in TBST and developed with diaminobenzidine substrate (with cobalt chloride as metal enhancer).

A separate membrane was immunoprobed with pooled sera of two toddlers diagnosed with allergy to wheat (dilution 1:3 with TBS-T) and incubated with anti-human IgE secondary antibodies conjugated with horseradish peroxidase. Other stages were conducted as described above (toddlers sera were collected by trained medical personnel with permission of local Ethical Board, after written informed consent of toddlers' legal guardians).

Separate membrane was stained with Ponceau-S and band of interest was excised and submitted for sequencing.

Mass spectrometry and protein sequencing

N-terminal protein sequence analysis was performed at BioCentrum Ltd. facility (Kraków, Poland). The sequentially detached phenylthiohydantoin derivatives of amino acids were identified using Procise 491 (Applied Biosystems, Foster City, CA, USA) automatic sequence analysis system working according to a standard protocol of its manufacturer. Protein for sequencing was excised from PVDF membrane after Western-blotting. Homology search was performed with Uniprot BLAST database [http://www.uniprot.org/].

Mass spectrometry analysis was performed with ESI--MS apparatus (Bruker Daltonics) on protein fraction previously purified by ammonium sulphate precipitation (20-30% of saturation) and desalted with Wide Pore Butyl (C_4) SPE column according to manufacturers application protocol no. BI-001 (J.T. Baker).

Competitive ELISA

Microtiter plates (Corning) were coated overnight at 4°C with 1 μg per well of native (not glycated) wheat proteins in pH 9.6 carbonate buffer. After coating the plate was washed three times with Phosphate Buffered Saline with 0.1% Tween-20 (PBS-T) and blocked with gelatin (1 g/100 mL) for 30 min at 37°C and again washed three times. In the next step, 50 mL of competing antigen (*i.e.* glycated protein or control sample) were added in series of double dilution starting from 2 mg/mL, followed by the addition of 50 μL of 70 μg/mL rabbit polyclonal antibodies to each well. After one hour of incubation at 37°C, the plate was washed three times with PBS-T and incubated with secondary antibodies (HRP conjugated, dilution 1:50,000 in PBS) for one hour, followed by threefold washing and 30 min of incubation with substrate (OPD). The reaction was stopped with 3 mol/L HCl and absorbance was read at λ=492 nm (ASYS UVM 340 microplate reader). The assays were done in triplicate. Results were expressed as percent of cross-reactivity calculated from EC50 of antigen binding curve for sample (glycated or control) and native protein.

Free amino group determination

The quantity of free amino groups was determined with the *o*-phthaldialdehyde (OPA) method according to method by Spellman *et al.* [2003]. Protein samples were dissolved in deionized water in 1 mg/mL concentration. Results were expressed as percentage of free amino groups of native sample, which was assumed as 100%. The assays were done in triplicate.

Statistical analysis

Where applicable, the results were statistically analysed with one-way ANOVA using GraphPad Prism software (GraphPad Prism 4.05 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

Electrophoretic separation of the salt-soluble proteins from wheat shows 17 bands in molecular weight range from 5 to 200 kDa. As it can be seen in Figure 1, dry heating has a significant influence on electrophoretic pattern of wheat proteins, regardless of the presence of glucose. However, the proteins subjected to dry heating with glucose show greater changes (qualitative and quantitative) in the electrophoretic pattern, with the most prominent effect of bands increasing their apparent molecular weight by approx. 2 kDa (the observed effect was consistent and repeatable; (Figure 1, dotted line provided for easier comparison). Western blotting with both allergic sera (IgE) and polyclonal rabbit antibodies (IgG) (Figure 1) shows that wheat proteins retain

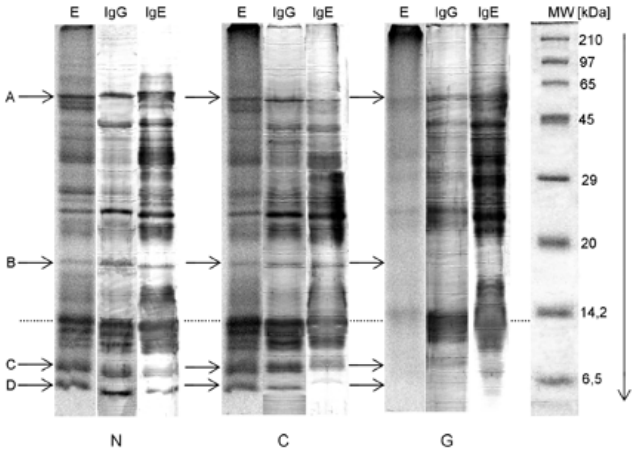

FIGURE 1. SDS-PAGE stained with Coomassie Brilliant Blue (CBB), Western immunoblotting with allergic sera (IgE) and Western immunoblotting with rabbit polyclonal antibodies (IgG) and of native protein (N), dry-heat glycation (G) and control sample (C). (MW – molecular weight standard).

most of their immunoreactivity after dry heating without glucose but glycation has much more impact on the properties of the protein. Immunoblotting with allergic sera shows 9 major and 5 minor reactive fractions in both a native and a control sample. The glycated sample shows partial loss of reactivity for both IgG and IgE of fractions with molecular weight of ~60 kDa (Figure 1, arrow A) and low molecular weight fractions of approx. ~6 and ~8 kDa (Figure 1, arrows D and E). Partial loss of IgG reactivity is also visible for ~24 kDa fraction, although IgE reactivity remained the same (Figure 1, arrow B). Practically total loss of reactivity of ~19 kDa fraction (Figure 1, arrow C) could be due to degradation, since protein band on electrophoresis lane could also not be seen.

Two-dimensional electrophoresis of native and glycated wheat proteins is shown in Figure 2. Although the electrophoretic image of the glycated proteins is hard to analyse and compare to that of the native ones, one of the major differences between the native and the glycated proteins is a significant shift of the isoelectric point of the glycated proteins towards low pH.

A quantitative analysis with competitive ELISA (Figure 3) has shown that dry heating caused a significant increase in immunoreactivity of the wheat proteins, while the increase caused by glycation was much lower (although statistically insignificant in comparison with both the control and the native protein).

OPA assay has shown a significant decrease in free amino groups content in glycated protein and an increase in the control sample (Figure 4).

A protein band with molecular weight of approximately 13 kDa (by SDS-PAGE), highly immunoreactive and susceptible for glycation, was selected for the N-terminal sequence analysis and resolved as: GPWLMPFPGQ. The Uniprot BLAST search has shown that the resolved sequence is in 90% similar to the sequence from alpha-amylase inhibitors from *Triticum aestivum*. A single amino acid difference was found to be proline (resolved sequence) in place of cysteine

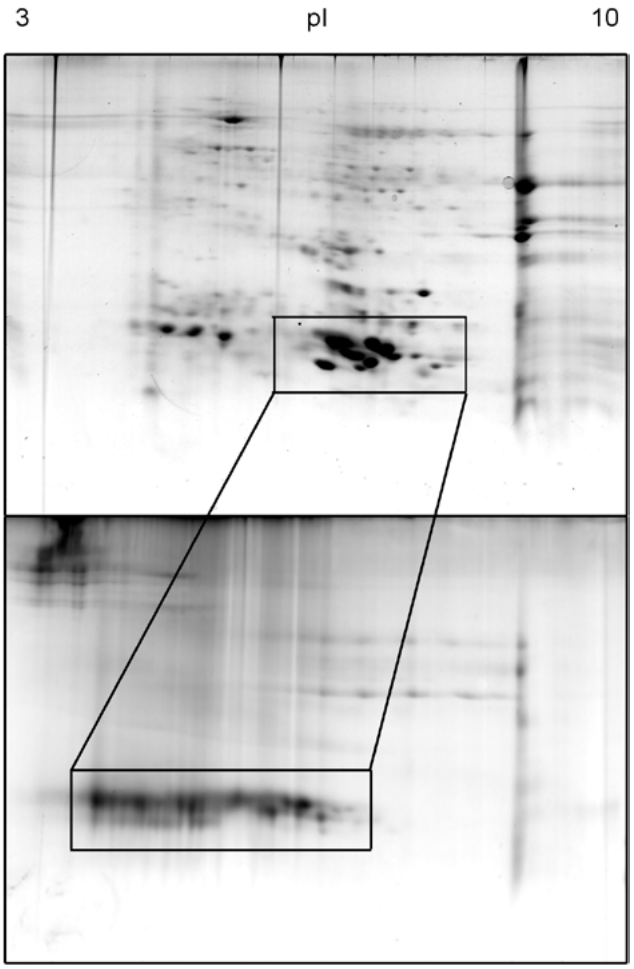

FIGURE 2. Two-dimensional electrophoresis of native (above) and glycated (below) wheat salt-soluble proteins.

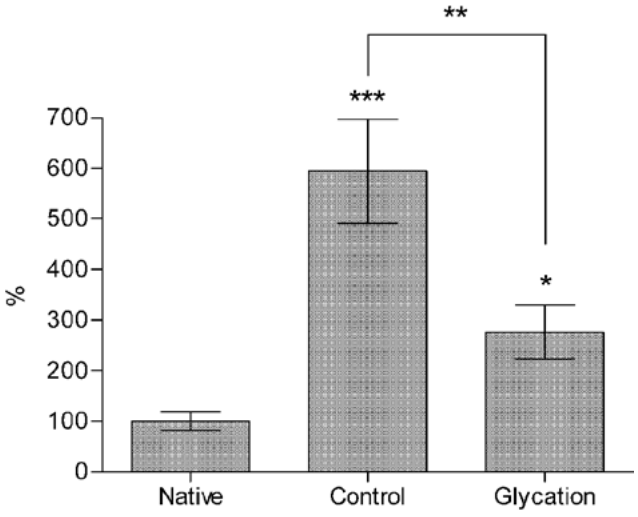

FIGURE 3. Immunoreactivity of native protein in comparison with glycated and control samples measured by competitive ELISA.

(database), which is the result of sequencing of protein with underived cysteine residues. The molecular weight of a dimeric alpha-amylase inhibitor was also determined by mass spectrometry and found to be approx. 15.6 kDa (Figure 5).

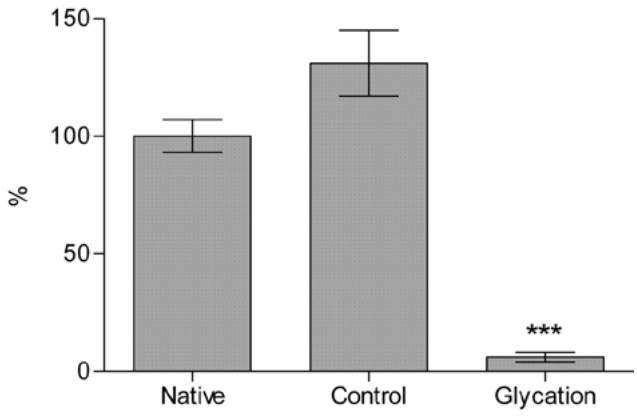

FIGURE 4. Comparison of free amino group assay of native protein with control sample and glycation. Results are expressed as mean ± SD.

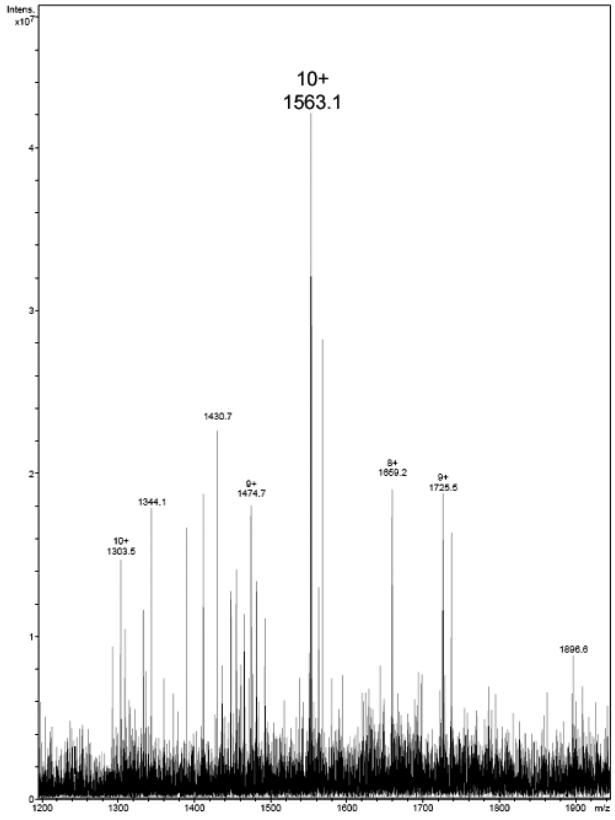

FIGURE 5. Mass spectrometry (ESI-MS) of purified highly immunoreactive, heat-resistant and glycation-susceptible protein. Main ion with +10 charge and molecular weight of 1563.1 Da equals to 15.6 kDa native protein.

DISCUSSION

Thermal processing may provide many beneficial effects to food, still it may also bring major changes in its immunoreactivity. Regular heating causes catastrophic disruption of a protein structure and should reduce immunoreactivity, although there are reports of the exact opposite, *e.g.* a patient with allergy to cooked but not raw fish [Davis *et al.*, 2001]. Thermal processing at the temperature of 55-70°C destroys tertiary structure of protein and may lead to reversible un-

folding and loss of secondary structure of one [Wal, 2003]. Dry heating has been reported to irreversibly destroy conformational epitopes of cherry allergen Pru av 1 [Sathe *et al*., 2005]. Thermal processing in the presence of reducing sugar may lead to glycation and – as expected – dry heating is a very potent but nondestructive method of protein glycation [Boratyński & Roy, 1998]. The observed shift in the molecular weight of the proteins by *ca.* 2 kDa can be explained as binding of 11–12 glucose molecules per one molecule of the protein. Such a high degree of substitution is possible for protein of sufficient lysine content (*e.g.* glycation of IgG in diabetes leads to conjugation of 5 to 25 molecules of glucose per a molecule of the protein [Lapolla *et al.*, 2000]) but for proteins with lower lysine content it may be connected with glycation of amino acids other than lysine *e.g.* arginine [Lederer *et al.*, 1999].

A significant increase in the immunoreactivity of the heat-treated wheat proteins (control sample) suggests that the majority of epitopes of those proteins are linear and the loss of their tertiary and secondary structures does not disrupt them. Moreover, it may even lead to their better exposure, as suggested by ELISA results for control group (Figure 3). This stands in agreement with the results of free amino group determination – the loss of tertiary and secondary structures leads to exposition of free amino groups that were hidden in the native proteins. In the case of the protein heat-treated with glucose, the amino groups – both present in the native protein and exposed by the loss of tertiary and secondary structures – were potential sites of glycation, which led to a significant loss of free amino group content. Moreover, conjugation of sugar moieties with protein may have masked some of the epitopes, thus reducing immunoreactivity of the glycated sample in relation to the control sample (while the result is still higher than for native protein). Substitution of positively charged amino groups with non-charged sugar moieties also leads to shift in the isoelectric point of the proteins, as shown by 2D-PAGE. Both conjugation with glucose and thermal treatment alone have changed (increased) the immunoreactivity of the proteins, however, the differences between the glycated and the native protein and glycated and control sample were significant. The presence of the glucose seems to "diminish" the effect of temperature on the immunoreactivity of protein, however, the introduction of new epitopes [Davis *et al.*, 2001] cannot be excluded.

The molecular weight determined by SDS-PAGE and the N-terminal sequence analysis suggest that the analysed protein is alpha-amylase inhibitor – either 0.19 [Uniprot P01085] or 0.53 [Uniprot P01084] (those proteins are of 94.4% sequence identity). This, however, stands in disagreement with the result of mass spectrometry, which shows that the analysed protein has molecular weight of approx. 15.6 kDa. Further database search has lead to the sequence of the wheat alpha amylase inhibitor with molecular weight of approx. 15.2 kDa [Uniprot Q4U1A0]. For this protein the obtained N-terminal sequence is preceded by 17 AA peptide with molecular weight of approx. 1.8 kDa while the sequence that follows has molecular weight of approx. 13.4 kDa. Knowing that the sequence analysis was performed on protein resolved by SDS-PAGE, transferred to a membrane and excised and mass spectrometry was performed on the purified native protein would suggest that the native form of the alpha-amylase inhibitor from wheat is a 15.2-15.6 kDa protein composed of two chains – a 13.4 kDa protein and non-covalently bound 1.8 kDa peptide.

CONCLUSIONS

1. Wheat proteins glycated by dry-heating change their electrophoretic mobility in a repeatable and observable manner.

2. Glycation by dry-heating caused molecular weight increase up to *ca.* 2 kDa which corresponds to 11-12 molecules of glucose per one molecule of the protein.

3. Dry-heating may increase IgG-reactivity of wheat proteins while glycation by dry-heating seems to have a smaller effect.

4. Glycation is able to reduce both IgG- and IgE-reactivity of some of wheat proteins, although the effect is dependant on the protein and not equal for both types of antibodies.

5. Highly immunoreactive, thermostable and susceptible to glycation protein with native molecular weight of approx. 15.6 kDa is most probably wheat alpha-amylase inhibitor.

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