



The influence of non-enzymatic glycosylation on physicochemical and biological properties of pea globulin 7S

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ABSTRACT

The research was aimed at defining the impact of non-enzymatic glycosylation of pea globulin 7S on its biological properties. Glycation of pea globulin 7S was carried out at the temperature of 37 °C for 7 days and at 60 °C for 3 days. No glycation of the of the pea globulin 7S was observed when using high pressure of 500 MPa. Polyclonic rabbit antibodies were produced for immunochemical studies. The immunologic activity of non- and glycosylated pea globulin 7S was examined with the ELISA and Western blot. Also, excretion of IL-5, -10 and 13 as well as INF- γ by human peripheral blood lymphocytes along with symptoms of allergy to soy proteins under the influence of non- and glycosylated pea globulin 7S was assessed, as well their apoptosis and necrosis. Moreover, transportation of those proteins by the Caco-2 monolayer and their cytotoxicity were also studied. The results have proved that pea globulin 7S glycosylated in various physicochemical conditions is characterized by different properties to those of non-glycosylated ones. Lysine glycation entails a decrease in its nutritional value. Glycosylated pea globulin 7S manifests lowered affinity to antibodies, which suggests a limitation of its immunoallergic properties. On the other hand, the fact that glycosylated pea globulin 7S stimulates maturing of Th0 cells to Th2 ones, confirms its role in the process of food allergy induction. Decreased cytotoxicity of glycosylated pea globulin 7S and its transportation through the Caco-2 monolayer may stand for its allergenicity caused by its limited transportation to the cells of the immune system.

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1. Introduction

An increased incidence of food allergies to milk and soy proteins entails an increased interest in pea proteins viewed as a potential source of proteins for e.g.: production of baby formulas (Davidson, 2001). This results from the fact that the pea seed includes only two allergenic proteins – vicilin (Pis s) and convicilin (Pis s 2) – when compared to six allergenic proteins in soy or five ones in milk (www.allergen.org – IUAS/WHO).

Pea proteins are divided into two basic subunits: globulins 11S, called legumins, and globulin 7S, called vicilins. Altogether, they make for 60–70% of all proteins in the pea seed. Proportions between legumins and vicilins are highly changeable, depending on the cultivated pea variety (cultivar) and span from 0.5 to 4.0. It means that the content of globulin 7S may be 22–52% (35% on average), and the content of globulin 11S from 13% to 43% of the total protein amount (Pedrosa, De Felice, Trisciuzzi, & Ferreira, 2000). The remaining 30–40% of proteins in pea seed consists of albumins – mostly enzymatic proteins, lectins, together with protease and amylase inhibitors.

Pea vicilin (globulin S7) is a homotrimer and its molecular weight is 150 kDa. It consists of three identical subunits and the molecular weight of each of them is 50 kDa. They do not have cysteine residues (Gatehouse, Lycett, Croy, & Boulter, 1982). Newly synthesized units of vicilin are subjected to glycosylation in endoplasmic reticulum, after which they are transported to aleurones where their trimer is formed. The homotrimer is subjected to post-translational enzymatic hydrolysis (so-called ‘nicking’), which, however, does not cause its cleavage (Chrispeels & Greenwood, 1987; Gatehouse et al., 1982; Lycett et al., 1983). Vicilin manifests a high homology of its amino-acid sequence to Ara h 1 peanut globulin 7S, and Len c 1 lentil allergen (www.allergome.org).

Food processing in the course of production and preparation for consumption may lead to structural and functional changes to the contained food proteins (Davis, Smales, & James, 2001). Non-enzymatic glycosylation, called glycation, is one of such reactions, commonly occurring in the aforementioned processes. The effects of this reaction vary from changes to the protein solubility, through a loss of nutritional value and an increase of antioxidation potential, to formation of AGEs (Advanced Glycation End-Products), or sometimes even formation of neoallergens (Achouri et al., 2006; Chevalier, Choberta, Popineau, Nicolas, & Haertle, 2001; Davis et al., 2001; Lagemaat van de, Silvána,

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Moreno, Olano, & del Castillo, 2007; Li, Enomoto, Ohki, Ohtomo, & Aoki, 2005; Wal, 2003; Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). Glycation is a two-step process, the former of which leads to formation of Schiff bases and is a reversible process. The latter, applying the Amadori rearrangement, produces ketoamine, which is a stable product (Shapiro, McManus, Zalut, & Bunn, 1980). In anaerobic environment, the Amadori products are rearranged to deoxyglucosons, which leads to regeneration of free lysine, however, in aerobic environment, they cleave glucoson in the process of auto-oxidation. The formed dicarbonyl compounds may react not only with free amino residues from lysine, but also with cysteine, histidine, and tryptophan (Thorpe & Baynes, 2004). Moreover, the proteins may be subjected to cross-linking by lysine and arginine (Lederer & Buehler, 1999). Glycation may occur in technological processes during lyophilisation (Zheng, Wu, & Hancock, 2006), drying (Oliver, Melton, & Stanley, 2006), cooking (Foerster & Henle, 2003), microwaving (Noma et al., 2009), and roasting (Chung & Champagne, 2001). Moreover, food processing can induce some covalent modifications of proteins resulting in various post-translation reactions, such as phosphorylation, glycosylation, proteolysis, deamidation, cross-linking, disulphide bonding cyclisation, and glycation (Mills, Sancho, Moreno, & Kostyra, 2007).

The aim of this research was to define the influence of non-enzymatic glycosylation of pea globulin 7S and its interaction with glucose resulting from high pressure conditions on its biological properties such as conformational changes, immunoreactivity, cytotoxicity, transportation through the Caco-2 monolayer, and necrosis and apoptosis of lymphocytes.

2. Methods

Flour made of 'Princess' variety pea seed has been used as the study material. It was obtained from the Institute of Food Research in Norwich (United Kingdom).

2.1. Extraction of globulin 7S from the pea (Croy, Gatehouse, Tyler, & Boulter, 1980; Gatehouse, Croy, Morton, Tyler, & Boulter, 1981; Lambert, Chambers, Phal, & Wright, 1987)

10 g of pea flour was suspended in 100 mL of phosphate buffer, pH 7.3, containing 0.9% of NaCl. The suspension was agitated for 60 min at the temperature of 20 °C by an agitator. The sediment was centrifuged at 15,000×g at the temperature of 4 °C. The supernatant was salted with ammonium sulfate. The first salting was carried out by adding small portions of ammonium sulfate until the level of saturation reached 50%, mixing the solution with a magnetic stirrer. The precipitated sediment was centrifuged at 15,000×g for 5 min at the temperature of 4 °C. The supernatant was salted again until it reached the 90% saturation level and the obtained sediment was centrifuged in conditions identical to the aforementioned ones. The obtained supernatant was salted until it reached the 100% saturation level and the obtained sediment was centrifuged and lyophilized. The content of the protein was marked with the BCA method using a reagent set by Pierce according to the producer's instructions.

2.2. Electrophoresis in denaturing conditions on the polyacrylamide gel (SDS-PAGE)

The electrophoresis in denaturing conditions on the polyacrylamide gel (SDS-PAGE) was carried out in Laemmli buffer system using a gel sized 8×8 cm and 1 mm thick. Its total acrylamide concentration (%T) was 12%, and N,N'-methylenebisacrylamide concentration (%C) was 2.6% at the stabilized voltage of 100 V. 5 µL of sample buffer (Sigma S3401) was added to a 5 µL sample with 1 mg/mL protein concentration. Then, it was incubated at the temperature of 95 °C for 5 min and placed on the gel.

2.3. Glycation of globulin 7S

The purified globulin 7S was subjected to glycation in various conditions:

- glycation at the temperature of 37 °C (310 K);
- glycation at the temperature of 60 °C (333 K); and
- glycation in high pressure (room temperature, 500 MPa).

2.4. Glycation at the temperature of 37 °C (310 K)

The examined protein was subjected to glycation at the temperature of 37 °C for 7 days. 1 mg of purified globulin 7S was dissolved in 1 mL of saline phosphate buffered (PBS) adding 1 mg of glucose. To provide microbiological safety of the examined protein, 0.05% of sodium azide was added. The examined solution was placed in closed vials and incubated in a holding oven at 37 °C for 7 days. On finishing the glycation process, a 2-hour dialysis in PBS was carried out in order to remove the glucose.

2.5. Glycation at the temperature of 60 °C (333 K)

Glycation was carried out at 60 °C for 3 days. 1 mg of purified globulin 7S was dissolved in 1 mL of saline phosphate buffered (PBS) adding 1 mg of glucose, and 0.05% of sodium azide and then the sample was lyophilized. The lyophilized sample was placed in closed vials and incubated in a holding oven at 60 °C for 3 days. On finishing the process, the samples were PBS-dissolved and dialysed at 4 °C for 24 h against redistilled water and then lyophilized.

2.6. Glycation in high pressure

Purified globulins 7S were subjected to high pressures using the Unipress device. The solutions, which contained 1 mg of globulin 7S and 1 mg of glucose in 1 mL of saline phosphate buffer (PBS), were placed in polytetrafluoroethylene containers and subjected to the pressure of 500 MPa (~4934 at.) for 15 min at room temperature. The glucose was removed by 24-hour dialysis against PBS.

2.7. UV spectrum

Measurements of UV spectra were taken by the NanoDrop 1000 spectrophotometer (Producer – Thermo Fisher Scientific, USA). 2 µL of the pea protein extract was placed in the chamber of the spectrophotometer. UV spectra were obtained using the software computer Protein A 280.

3. Identification of glycation products

3.1. Identification of fructosamine content (Lloyd & Marples, 1984)

The fructosamine content was identified by the microplate method using a solution of nitro blue tetrazolium (0.25 mM) in a carbonate buffer (0.1 M, pH 10.8). 8 µL of carbonate buffer and then 250 µL of reagent were added to the solution that contained 1 mg/mL of the examined protein. The mixture was incubated at 37 °C and after 10 and 15 min of incubation two absorbance measurements were taken at the wavelength of 530 nm. The measurements were subtracted from each other and the content of fructosamine was expressed as a percentage in relation to the purified globulin 7S.

3.2. Identification of free amino groups with OPA method (Nielsen, Petersen, & Dammann, 2001)

1 mg of globulin 7S was dissolved in 1 mL of water. 125 µL of the sample were collected and the mixture of the following composition

was added: 0.8 M of orthophthalic dialdehyde, 0.6 M of methanol, 0.06 M of β -mercaptoethanol, and 1 M of sodium tetraborate. The samples were incubated at 25 °C for 20 min, and then the absorbance measurement was taken at the wavelength of $\lambda = 340$ nm in relation to a blank test that did not contain any proteins. The contents of free amino groups were expressed as a percentage in relation to the native protein.

3.3. Identification bound sugar with the anthrone method (Laurentin & Edwards, 2003)

Samples with the protein concentrations of 100 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ were prepared by addition of 200 μL of saline phosphate buffer (PBS). On placing the test tubes on ice, 55 μL were collected from the each sample. 275 μL of anthrone reagent (160 mg of anthrone dissolved in 10 mL of 14 M H_2SO_4) were added to each of them and the mixtures were incubated at 100 °C for 10 min. Then, the examined samples were cooled to the temperature of 25 °C and another absorbance measurement was taken at the wavelength of 620 nm. The results were read from the calibration curve charted for glucose in its concentration span from 0 to 100 $\mu\text{g/mL}$ of PBS.

3.4. Immunization and obtaining the antibodies

Three one-year-old rabbit females, body weight of 2–3 kg, were used for vaccinations (The Ethical Committee Permission No.53/2003/N). 1 mg of purified globulin 7S was dissolved in 0.5 mL of saline solution and 0.5 mL of Freund's adjuvant was added. On formation of the emulsion, 0.5 mL of the mixture was applied subcutaneously. Freund's complete adjuvant was used for the first dose and the following ones were prepared with the incomplete adjuvant (Sigma-Aldrich). Four subsequent vaccinations were performed in 21-day intervals. The course of immunization process was controlled by ELISA using sera obtained from the rabbit blood. Ten days after the last dose of vaccination, the rabbits were exsanguinated. The collected blood was coagulated for 1 h at 37 °C and centrifuged twice at 5000 $\times g$ for 15 min to collect the serum. The antibodies were obtained by salting with ammonium sulfate until the saturation level of 50%.

4. Immunoenzymatic tests (ELISA)

4.1. The 'simple' test

Activities of the polyclonal rabbit antibodies were assessed by the immunoenzymatic test (ELISA). The plates were flattened with the purified globulins 7S for 24 h at the temperature of 4 °C, in the amount of 100 μL of protein solution with the concentrations from 10 to 50 μg of protein to 50 mM of carbonate buffer with pH of 9.6. Then, the free places were saturated with the 1% solution of gelatine dissolved in PBS for 30 min at 37 °C.

The examined rabbit sera were diluted from 1:50 to 1:51,200, and the purified antibodies were diluted from 1 mg/mL to 0.0001 mg/mL and incubated for 1 h at the temperature of 37 °C. Then, secondary antibodies marked with horseradish peroxidase (Sigma Cat. No: A9169, 100 μL diluted 1: 5000 in PBS-Tween) were applied and rinsed. After that, 0.4 mg of o-phenyldiamine in mL of 50 mM of a citrate-phosphate buffer, pH 5.0, with an addition of hydrogen peroxide, was added. After 30 min, the reaction was stopped with 5 M of HCl and the absorbance was measured with a microplate reader (ASYU UVM 320) at the wavelength of $\lambda = 492$ nm. The antibody titer was defined as dilution at the EC_{50} point of a sigmoid curve (using the GraphPad Prism 4.05 application).

4.2. The 'competitive' test

To assess the immunoreactivity of the examined protein (globulin 7S) a competitive immunoenzymatic test was carried out (the

competitive ELISA). The plates were flattened as above. Then, the wells were supplemented with the rabbit globulin 7S antibodies in the amount defined using the simple test, as well as the examined sample (the competitor) in dilutions that ranged from 1 to 0.00001 $\mu\text{g/mL}$, and incubated for an hour at the temperature of 37 °C (310 K). A positive test (100% of the signal force) was made by the wells with no addition of the competitor; the negative test was made by the wells with no addition of globulin 7S antibodies. Further activities were performed as above.

Using sigmoid curves, the EC_{50} (the amount of an antigen necessary to a 50% signal decrease) values were read and a percentage of cross-reactions using the following formula:

$$\text{CR}\% = (\text{EC}_{50}\text{N}/\text{EC}_{50}\text{C}) \times 100\%$$

where CR% – a percentage of cross-reactions, EC_{50}N – a concentration of specific antigen necessary to bind 50% of available antibodies, C_{50}C – a concentration of cross-reacting antigen necessary to bind 50% of available antibodies.

4.3. Western-immunoblotting

Electro-transfer was performed in a semi-dry system. A gel plate from the preformed electrophoresis, a Whatman 3 blotting paper and a Immobilon PSQ 0.22 μm membrane (previously activated with methanol) were immersed in a Dunn's transfer buffer that consisted of 10 mM of NaHCO_3 , 3 mM of Na_2CO_3 , 20% (v/v) of methanol, pH 9.9, and then they were placed in a transfer device. The transfer was carried out for an hour, with the intensity of 0.8 mA/cm^2 of the gel surface. On finishing the transfer, the membrane was incubated for 60 min in a Tris buffer, pH 7.5, with an addition of 0.9% of sodium chloride and 0.1% of Tween-20 (TBS-T). Then, the membrane was incubated again in a solution of purified globulin 7S antibodies in the concentration of 0.1 mg/mL (in TBS-T). After the incubation, the membrane was rinsed three times in TBS-T. On performing those activities, another incubation of the membrane was performed – in the solution of secondary antibodies conjugated with horseradish peroxidase. After rinsing the membrane, it was developed with diaminobenzidine (DAB) according to the producer's instructions. The developed membrane was dried out at the temperature of 37 °C and read by a scanner.

5. In vitro examinations of the isolated peripheral blood lymphocytes

5.1. Isolation of mononuclear human peripheral blood cells

The blood for the experiment originated from people allergic to soy proteins. A permission to collect blood from the volunteers was granted by the Bioethical Committee at the Warmia and Mazury Doctors' Chamber. The blood was collected into heparinized vacuum tubes and lymphocytes were isolated by Histopaque 1077 (Sigma) according to the producer's instructions. The cells were suspended in the medium of the following composition: 98% of RPMI-1640, 1% of human serum AB, 1% of gentamicin. Then, they were cultured for 24 h on 24-well cultivation plates in the amount of 1 million of cells per a well. The culture took place at the temperature of 37 °C (310 K) in an incubator, in the 5% CO_2 atmosphere and 95% of relative humidity. After the 24-hour culture, the wells were supplemented with solutions containing the proteins glycosylated in various environments and a solution of the native peptide, until the concentration of 20 $\mu\text{g/mL}$ was reached. Then, the culture was continued for the next 7 days.

5.2. Measurement of interleukins excreted by the human peripheral blood lymphocytes

After 7 days of cell incubation with the soy-extracted globulin 7S, 100 μ L of the medium were collected from each culture well to measure concentrations of inflammatory processes mediators. Concentrations of the following interleukins were identified: IL-5, IL-13, IL-10, IL-17, and INF- γ . The interleukins were measured by the flow cytometry with fluorescent detection using a Cytometry Bead Array System (CBA) by BD Biosciences.

5.3. Examination of apoptosis of human peripheral blood lymphocytes by the flow cytometry

After 7 days of cell culture in the presence of the allergen, the cells were taken from the bottom of the plate and collected into Eppendorf tubes. The suspension was centrifuged at 500 $\times$ g for 10 min at 4 °C, after which the supernatant was separated and the cells were suspended in a buffer with an addition of 2% (v/v) annexin V. The cells were incubated for 15 min on ice in the dark, and then centrifuged at 300 $\times$ g for 7 min at 4 °C. The supernatant was separated and the cells were suspended in 200 μ L of annexin buffer and 2 μ L of propidium iodide diluted in 1:1000 was added. The measurements were taken in a flow cytometer (FACS by BDBiosciences, NY, USA).

5.4. Cell culture

Caco-2 cells were cultured according to method described by Iwan et al. (2011).

5.5. Transport studies

After measuring the monolayer integrity, the Caco-2 monolayers were rinsed two times with HBSS. The transport of pea globulin 7S has been determined from apical to basolateral (A–B) direction. After incubation for 30 min, the Hank's solution was replaced with a native or modified pea globulin 7S solution (400 μ L on the apical side) to the final concentration of 1 mg/mL of the investigated globulin. Samples of the basolateral solutions were taken after 3 h. The concentration of pea globulin 7S in the basolateral solutions was determined using the ELISA method.

5.6. Assessment of the PBMC proliferation using the WST-1 proliferation test

After 12 h of PBMC incubation with 100 μ L of native or modified pea globulin 7S (500 μ g/mL), the WST-1 proliferation test (Roche Diagnostic) was added in the ratio 1:10 and the incubation was continued for 150 min. The cell incubation time for all the investigated samples and for the WST-1 test was determined in previous experiments. The absorbance was measured at the wavelength of λ = 450 nm using an ELISA reader (Biogenet Asys UVM 340).

6. Results and discussion

The SDS-PAGE electrophoresis under reduction conditions of pea protein extract, native pea globulin 7S, glyated globulin 7S at 37 °C and 60 °C, pea globulin 7S treated by high pressure in the presence of glucose and Western-immunoblotting of all samples are presented in Fig. 1. The electrophoretic separations of the pea protein extract and native pea globulin 7S show 17 and 8 bands in molecular weight ranges from 12 to 95 kDa, and from 12 to 50 kDa, respectively. These results are consistent with ones by the other authors (Croy et al., 1980; Gatehouse et al., 1981; Lambert et al., 1987). As it can be seen in Fig. 1, heating of native pea globulin 7S in the presence of glucose has a significant influence on its electrophoretic pattern. Glycation

of pea globulin 7S at 37 °C resulted in the disappearance of the bands with molecular weight about 22 kDa. In the case of native pea globulin 7S at 60 °C 4 bands with molecular weight about 20, 29, 30 and 50 kDa were observed, which characterizes a significant lower intensiveness. The observed electrophoretic changes of the native and heated pea globulin 7S in the presence of glucose can be explained by analyzing the glycation process. Glycation of proteins is a spontaneous reaction with complex kinetics that depends on the type of protein, reduction sugar, temperature, and pH (Yeboah, Alli, & Yaylayan, 1999; Pedrosa et al., 2000; Cloos & Christgau, 2002; Seidler & Yeargans, 2002; Thorpe & Baynes, 2004; Kostyra, Wociór, Rudnicka, Rydzewski, & Kostyra, 2010). Protein glycation may affect the hydrophilic/hydrophobic balance and/or net charge at the protein surface, leading to changes in the protein–solvent and protein–protein interactions. These, in turn, may lead to changes in folding, stability or biological activity of glyated proteins (Pedrosa et al., 2000). When discussing the protein-glyated protein interactions, it must be taken into account that pea globulin 7S is a trimer of 50 kDa subunits. Some of the pea 50 kDa subunits in pea globulin 7S are nicked by posttranslational proteolysis soon after biosynthesis, and the resulting peptides with molecular weights from 12.5 to 33 kDa remain associated with the intact subunits in the native 150 kDa oligomer (Gatehouse et al., 1982; Pedrosa & Ferreira, 1994). In addition, during of glycation of pea globulin 7S, its partial denaturation occurs. According to Seidler and Yeargans (2002) glycation of denatured proteins undergoes more readily than glycation of native proteins, which suggests that native conformation provides protection against chemical modification by glyating agents. The additional confirmation of the pea globulin 7S glycation at 37 °C and 60 °C is provided by the results for contents of free amino groups in pea globulin 7S subjected to glycation: they are about 25% (G-60) and 34% (G-37) lower when compared to their contents in native pea globulin 7S (Fig. 2). In the case of fructosamine these results were 50% (G-60) and 135% (G-37). The presence of fructosamine in the reaction mixture is also a confirmation of glycation occurrence (Mosca et al., 1987). The UV spectra of native pea globulin 7S and the glyated one at 37 °C and 60 °C are presented in Fig. 3. Pea globulin 7S glyated at 37 °C was characterized by two absorption bands in the ranges of 260–280 nm and 220–240 nm. Pea globulin 7S glyated at 60 °C was also characterized by two absorption bands, however, its spectrum manifested a significantly higher absorbance in the range of 220–320 nm. The UV spectra of glyated pea globulin 7 prove the conformational differences between native and glyated pea globulin 7S (Agrirova & Agrirov, 1999). The above results suggest that glyated pea globulin 7S probably differs from the native one in its functional properties such as solubility, emulsifying and foaming (Pedrosa et al., 2000). The high pressure can influence the conformational structure of proteins, causing the changes of their physico-chemical and biological properties (Chicón, López-Tandino, Alonso, & Belloque, 2007; Pedrosa & Ferreira, 1994). In our investigations we observed no changes in the electrophoretic mobility, the content of the free amino groups and fructosamine between native pea globulin 7S and the one treated with high pressure in the presence of glucose. This fact proves that pea globulin 7S does not undergo glycation in the applied conditions of high pressure. The slight increase in the content of free amino groups in the pressured pea globulin 7S is probably a result of inclusion complexes formation. Glucose trapped in them is not completely removed during dialysis.

The second part of the investigations was devoted to changing of the biological properties of native and glyated pea globulin 7S treated with high pressure in the presence of glucose. The influence of pea globulin 7S glycation on its immunogenicity may be assessed basing on the results of Western-immunoblotting and ELISA. All immunogenic glyated pea globulins 7S have their molecular weights ranging from about 12 to 50 kDa. Glyated pea globulin 7S (G-37) showed only two intensive immunogenic bands on the electrophoretic gel. They had molecular weight of about 12 and 50 kDa. The other bands

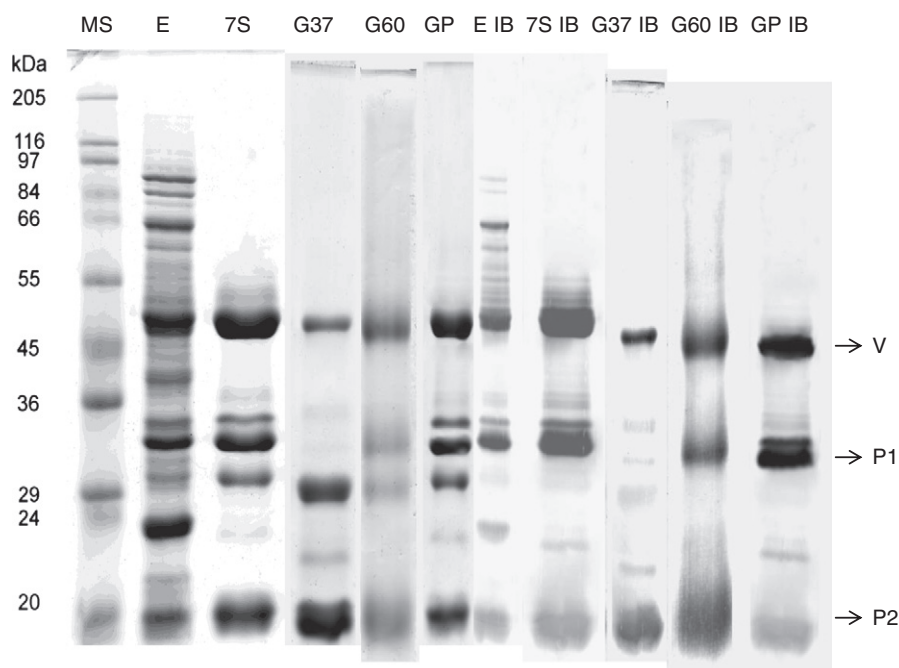


Fig. 1. SDS-PAGE patterns of native and treated pea proteins. Lanes: MS, molecular mass marker (kDa); E, protein extract; 7S, pea globulin 7S, control sample (without glucose); G37, pea globulin 7S treated in the presence of glucose at 37 °C for 7 days; G60, pea globulin 7S treated in the presence of glucose at 60 °C for 3 days; GP, pea globulin 7S treated in the presence of glucose by high pressure (500 MPa for 15 min); EIB, Western-immunoblotting of the pea protein extract (E); 7SIB, Western-immunoblotting of the pea globulin 7S; G37IB, Western-immunoblotting of the pea globulin 7S glycated at 37 °C for 7 days; G60IB, Western-immunoblotting of the pea globulin 7S glycated at 60 °C for 3 days; GPIB, Western-immunoblotting of the pea globulin 7S treated in the presence of glucose by high pressure (500 MPa for 15 min).

showed a very weak intensity (Fig. 1, G-37IB0). All glycated globulin 7S (G-60) showed an additional band with the molecular weight of about 30 kDa. The investigation findings for the immunoreactivity of pea

glycated globulin 7S obtained by electrophoresis are confirmed by the ones obtained with ELISA (Fig. 4). Generally, it can be concluded that glycation of pea globulin 7S decreases its immunoreactivity. The fact that glycation of pea globulin 7S at higher temperatures slightly decreases its immunoreactivity when compared to the glycation at lower ones is interesting. This phenomenon can be explained by formation of a different conformational structure of pea globulin 7S during glycation at different temperatures. The fact that different amounts of amino acids are glycated at different temperatures cannot be excluded. (Frączek, Kostyra, & Kostyra, 2008; Spencer, Chandler, Higgins, Inglis, & Rubira, 1983; Wensing et al., 2003). As a matter of fact, glycation of pea globulin 7S in the environment of glucose and high pressure did not cause any changes to its immunoreactivity, because the increase observed immediately after the exposure to high pressure disappeared after some time. The stimulation of excretion of interleukins IL-5, IL-10, IL-13, and IFN γ by human peripheral blood lymphocytes in a person with an allergy to soy proteins under the influence of the native and glycated pea proteins at 37 °C and 60 °C, and treated with high pressure

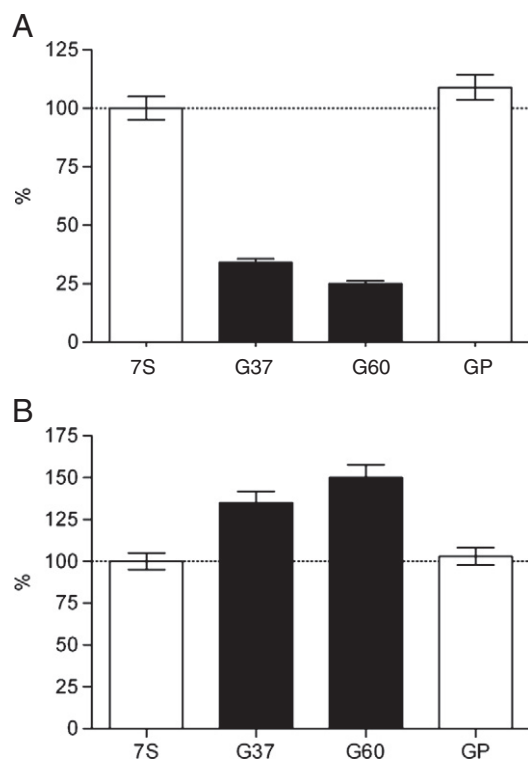


Fig. 2. A and B. Contents of free amino groups (A) and fructosamine (B). 7S – non-modified globulin 7S. G37 – globulin 7S glycated at 37 °C 7 days. G60 – globulin 7S glycated at 60 °C for 3 days. GP – globulin 7S glycated in high pressure (500 MPa for 15 min).

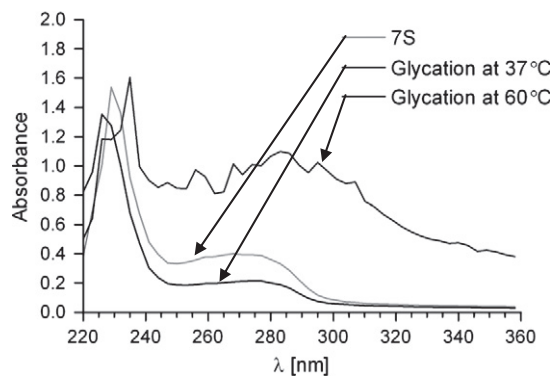


Fig. 3. UV spectra of the pea globulin 7S, globulin 7S glycated at 37 °C 7 days, and globulin 7S glycated at 60 °C for 3 days.

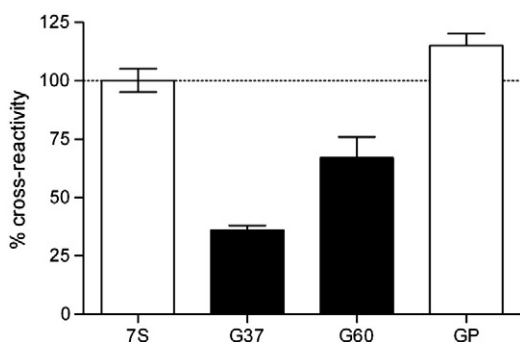


Fig. 4. Changes to the immunoreactivity of the purified pea globulin 7S (7S), globulin 7S glycosylated at 37 °C 7 days (G37), globulin 7S glycosylated at 60 °C for 3 days (G60), and globulin 7S glycosylated in high pressure (GP).

(500 MPa) in the presence of glucose is presented in Fig. 5A–D. The stimulation of interleukins and interferon γ excretion was performed using peripheral blood lymphocytes from a person with an allergy to soy proteins, due to a lack of one with an allergy to pea proteins. Such a study model also seemed interesting because no data on cross-reactions between soy and pea proteins have been found in the literature. The problem is important because these proteins are characterized by a relatively high similarity of their amino acid sequences (40–50%) (the Uniport/ExPASy database). The analysis of the results presented in Fig. 5 shows that all glycosylated pea globulins 7S stimulated the excretion of the examined cytokines to various degrees. Glycosylated pea globulin 7S (G-37) stimulated excretion of all the examined interleukins and IFN γ in a much stronger way than glycosylated pea globulin 7S (G-60). This means that glycosylated pea globulin 7S encourages lymphocytes to excrete interleukins stimulating B cells to produce antibodies IgE (IL-13) and IgA (IL-5) as well as the ones that activate macrophages and strengthen phagocytosis (IFN γ). Glycosylated pea globulin 7S also triggers maturing of

THO cells to Th2 ones that are responsible for occurrence of allergic reactions (IL-10). It is noteworthy that IL-5 and IL-10 may play a pathogenic role in the cases of food hypersensitivity in allergic children (Maciorkowska, Dziecioł, Kemona, & Kaczmarek, 2000). Also, the influences of native or glycosylated pea globulin 7S on the amounts of lymphocytes that entered the state of necrosis or apoptosis was investigated. No statistically significant differences between all the examined samples were noticed for those both parameters. The level of apoptosis was relatively constant for all the samples and oscillated within 2–4% and for necrosis it was 6–9%. These results, however, are not presented. Fig. 6 shows the results of transportation of native and glycosylated pea globulin 7S through a monolayer of Caco-2 cells. Native pea globulin 7S was transported in the amount of circa $0.261 \mu\text{g} \times (\text{mg} \times \text{h} \times \text{cm}^2)^{-1}$. The amounts of the glycosylated pea globulin 7S at 37 °C (G37) and 60 °C (G60) transported through the monolayer of Caco-2 cells decreased to circa $0.02 \mu\text{g} \times (\text{mg} \times \text{h} \times \text{cm}^2)^{-1}$, respectively. Such a definitive limitation to the transportation of the glycosylated pea globulin 7S may be explained by a change to its hydrophobic–hydrophilic nature. Pea globulin 7S contains 30 lysine residues that may be subjected to glycosylation. It means that glycosylated pea globulin 7S is definitely more hydrophilic than the non-glycosylated one. What makes its consequence is a change to the conformational structure of pea globulin 7S that causes the limitation in its transportation through the Caco-2 monolayer. The results of the cytotoxic activity of native (7S) and glycosylated pea globulin at 37 °C (G37) and 60 °C (G60) towards the proliferation of Caco-2 cells are presented in Fig. 7. Native pea globulin 7S (7S) lowered the proliferation of Caco-2 cells with about 60% in relation to the culture medium ($p \leq 0.001$). Glycosylated pea globulin (G37) and (G60) lowered the proliferation of Caco-2 cells with about 22% ($p \leq 0.001$) and 7% ($p \leq 0.05$), respectively. It is likely, that the decrease to the proliferation of Caco-2 cells when adding the native pea globulin 7S was caused by its interactions with the ingredients of the culture medium that impede their utilization as nutritional elements during the cellular growth. However, the glycosylated pea globulin 7S could be an additional energetic

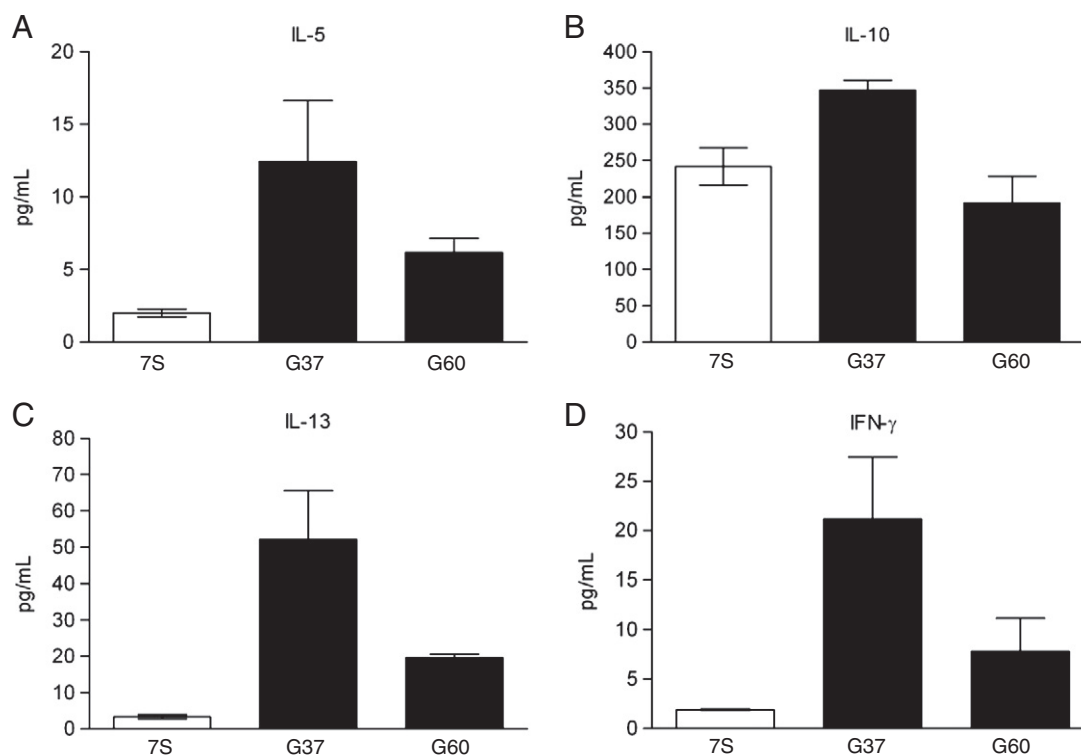


Fig. 5. A–D. Stimulation of excretion of interleukins IL-5, IL-10, IL-13, and IFN γ by human peripheral blood lymphocytes in allergic person to soy proteins under the influence of the native 7S and glycosylated at 37 °C (G37) and 60 °C (G60).

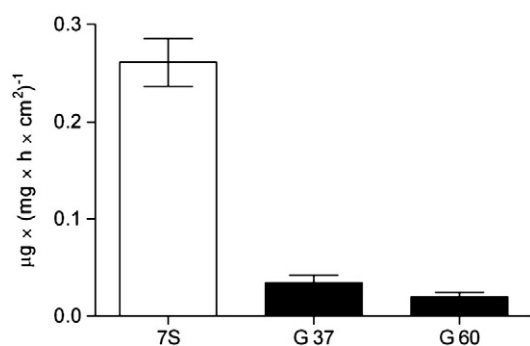


Fig. 6. Transportation of the purified globulin 7S (7S), globulin 7S glycated at 37 °C 7 days (G37), and globulin 7S glycated at 60 °C for 3 days (G60) through the Caco-2 monolayer.

material in a form of glucose attached to pea globulin 7S. Additionally, it is noteworthy that the proliferation of Caco-2 cells under the influence of native and glycated pea globulins 7S correlates with the transportation of these proteins through the monolayer of Caco-2 cells. Pea globulin 7S inhibits the proliferation of Caco-2 cells and its smaller amounts are transported through monolayer Caco-2 cells, while glycated pea globulin 7S (G60) stimulates the proliferation of Caco-2 cells and simultaneously transported through monolayer of Caco-3 cells in larger amounts. This fact may suggest that transport through a monolayer of Caco-2 cells is an active process.

7. Conclusions

The pea globulin 7S glycated in various physicochemical conditions is characterized by different biological properties when compared to the non-glycated one. Glycation of lysine entails a decrease in its nutritional value. The glycated pea globulin 7S manifests a lowered affinity to antibodies, which suggests a limitation of its immuno-allergenic properties. On the other hand, stimulation of maturing Th0 cells to Th2 ones by the glycated pea globulin 7S confirms its participation in the process of food allergy development. The decreased cytotoxicity and transportation through the Caco-2 monolayer of the glycated pea globulin 7S may confirm its lowered allergenicity caused by a limitation of its transportation to the cell of the immune system.

Acknowledgments

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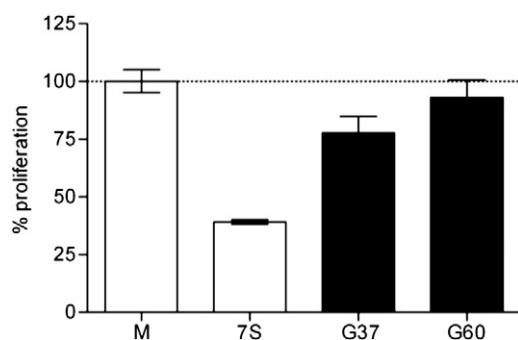


Fig. 7. Cytotoxic activity of the purified globulin 7S (7S), globulin 7S glycated at 37 °C 7 days (G37), and globulin 7S glycated at 60 °C for 3 days (G60) towards the Caco-2 cells.

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