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Effect of non-enzymatic glycosylation of pea albumins on their immunoreactive properties

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ABSTRACT

The aim of this study was to determine the effect of non-enzymatic glycosylation of pea proteins on their immunoreactive properties. Extracted total pea albumins were glycated. No changes were found in molecular weight distribution of total pea albumins before and after glycation using size exclusion chromatography and SDS–PAGE methods. SDS–PAGE GLYCO test stained the glycated proteins and OPA method showed 15% progress in glycation. Glycated and unglycated pea albumins were orally and intraperitoneally administered to Balb/C mice. Serum specific IgG and IgA and sIgA were determined. No difference in serum specific IgG level was found after oral mice immunization with TA and GTA. In the presence of antigen SPL lymphocytes culture showed higher proliferation activity as compared to the culture without the antigen addition. The glycation does not change the immunoreactivity of proteins significantly. During the presented route of immunization with TA and GTA specific tolerance mechanism could be induced.

1. Introduction

Each day a number of food proteins enter the gastrointestinal tract. They can provoke food intolerance or food hypersensitivity (Sampson, 2003).

It is considered that about 6% of infants population suffer from food allergy, and 2.5% of the allergenic infant population suffer from cow's milk allergy only (Sampson, 2003). The adult population suffer from food allergy to a lesser extent and statistically that is 1–3% (Zeiger, 1990). In the European countries about 3.3% of allergic population have allergy developed to beans, and 0.8% to green pea (Sell, Steinhart, & Paschke, 2005) and the green pea allergens appear to be an interesting area for research.

Pea is characterized by a high content of protein, dietary fiber, minerals, vitamins and antioxidants compounds (Alonso, Grant, & Marzo, 2001; Urbano et al. 2005) and pea proteins are of very high nutritional value due to amino acid composition, as they contain a lot of sulphur amino acids which are very important for mammals (Friesen, Kiarie, & Nyachoti, 2006). As green pea is an excellent and rich source of protein and belongs to family *Leguminosea* whose proteins are considered to be of high allergenic potential, it can be expected that pea proteins can readily induce allergenic reaction (Sathe, Kshirsagar, & Roux, 2005). Pea albumins have not been intensively investigated and pea allergens have been characterized to a lesser extent as compared to others (Lu, Queillien, & Popineau, 2000). Typical pea allergy is contributed to pea globulin fractions only. Malley, Baecher, Mackler, and Perlman (1975) showed that only albumins gave positive results in a skin test and determined molecular mass of green pea allergen as 11 kDa. Vioque et al. (1998) reported cytosolic albumin (PA2), which is not hydrolysed during germination, to be responsible for allergic reaction in chick-pea-sensitive individuals. Wang, Domoney, Hedley, Casey, and Grusak (2003) describe PA2 as potential allergen. Sell et al. (2005) examined allergenic properties of pea albumins fraction. All those work have demonstrate that in allergy to pea two albumin fractions PA1 and PA2 may be more allergenic than globulins.

Due to a high nutritional value of pea proteins sometimes they are modified to decrease their immunoreactive properties and use them as a food component. The aim of our study was to determine the effect of glycation of pea proteins on their immunoreactive properties. Glycation can spontaneously occur during a number of technological processes applied in industrial food processing, and is officially referred to as the Mailard reaction. Pea albumins were extracted from flour and purified, subsequently the process of glycation was performed.

Total pea albumins (TA) and glycated pea albumins (GTA) were orally and intraperitoneally administered to Balb/C mice. The level of specific immunoglobulin G (IgG) and A (IgA) in blood serum was determined as a humoral response factor. The solid phase enzyme linked immunospot assay (ELISPOT) and some tissue culture

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experiments were done to analyze cellular specific response. Lymphocytes from spleen (SPL) and mesenteric lymph nodes (MLN) were cultured with and without antigen and cell proliferation was observed under microscope. This paper describes the results obtained in the experiment.

2. Materials and methods

2.1. Materials and sample preparation

Pea seeds (*Pisum Sativum* L.) of Ramrod variety, were obtained from the Institute of Plant Farming in Łagiewniki (Poland). Seeds were milled in a WŻ-1 grinder (ZBPP Sadkiewicz Instruments, Poland) and sieved through a standard 0.40 mm sieve (NAGEMA, Germany).

2.2. Albumin extraction

Fifty grams of pea flour was extracted with 1000 mL H_2O d.d at 4 °C for 24 h, then the extract was centrifuged (22,500*g* at 4 °C for 15 min.). Pellet was discarded and supernatant containing total albumins was dialyzed against distilled water for 48 h. Then, the dialysate was lyophilized and so obtained the total pea albumin powder was further referred to as the total pea albumins (Higgins et al., 1986).

2.3. Proteins non-enzymatic glycosylation

TA (0.380 mg) and 0.780 g D(+) glucose (Sigma) were reconstituted in 20 mL sterile 0.10 M phosphate-buffered saline (PBS), pH 7.4 supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin and 50 μg/mL gentamicin. The bottle was closed in sterile and left at 37 °C for 7 days (Cazacu-Davidescu, Kostyra, Marciniak-Darmochwał, & Kostyra, 2005). Then, the solution was dialyzed against distilled water and concentrated by reverse dialysis against solid PEG–6000 (Sigma). Aliquots of glycated total pea albumins were frozen for other experiments.

2.4. Mice experiment

Balb/C (6- to 8-week old) mice were purchased from the Medical Research Centre, PAS, Warsaw. Mice were orally (on days 0, 1, 2, 10 or 0, 7, 14) or intraperitoneally (on days 0, 7, 14) immunized with 2 mg of TA, 2 mg of GTA in 250 μL of PBS and with 250 μL of PBS as a control group. Oral doses were administered with a 21-gauge gavage needle attached to a 1 mL tuberculin syringe directly to the stomach. Intraperitoneal immunization was done using tuberculin syringe with 0.4 needle. For immunization Complete Freud Adjuvant was used and for boosting incomplete. Total volume injected to mice was 200 μL.

The Local Care Use of Animals Committee (authorization 56/2006/N) approved animal handling and experimental procedures.

2.5. Sample collection

Serum samples from the mice were collected by saphenous bleeding. The blood was allowed to stand for 1 h at room temperature, then centrifuged at 12,000*g* for 10 min at 4 °C to separate serum. Then sera were collected and stored at −20 °C for later analysis.

Fecal samples were weighted and suspended at concentration of 100 mg/mL in PBS containing 0.1% sodium azide. Samples were stored at −20 °C for further analysis (Lee, Gierynska, Eo, Kuklin, & Rouse, 2003).

2.6. Protein determination

Protein was determined according to the Bradford's method (Bradford, 1976). Bovine serum albumin (BSA) (Sigma) was used as a standard protein.

2.7. SDS–PAGE gel electrophoresis

Polyacrylamide gel with a concentration of 15% was prepared according to the method described by Laemmli (1970). The separation of pea albumins was performed under constant current (24 mA), in Biorad electrophoretic equipment. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Sigma) and GelCode Glycoprotein Staining Kit (Pierce). Low molecular weight marker (LMWM) in the range from 6.5 to 66.0 kDa (Sigma) was used for molecular mass determination.

2.8. Fast protein liquid chromatography (FPLC)

Samples of TA and GTA were applied to a Superdex 75 column (HR 10/30; Pharmacia, Sweeden) at a flow rate of 0.5 mL/min. The chromatographic separation was performed in 0.025 M phosphate-buffer, pH 8.0. Elution profile was monitored by absorbance at 214 nm. The FPLC system was programmed by the use of FPLC Manager software (v.3.1). Molecular weight marker kit (in the range of 6.5–66 kDa) for gel filtration chromatography was used as a standard.

2.9. Quantitative determination of free α-amino groups in amino acids

The quantity of free amino groups was determined with the OPA method (Church, Swaisgood, Porter, & Catignan, 1983). OPA reagent was freshly prepared each time. OPA (160 mg) was dissolved in 4 mL of methanol, 0.4 mL of mercaptoethanol, and 200 mL of 0.1 mol L^{-1} sodium borate buffer. 0.125 mL of sample was added to 5 mL of *o*-phtalaldehyde (OPA) reagent. Blank sample did not contain albumin and was prepared identically. The absorbance was measured at 340 nm after 20 min. A calibration curve was obtained by using L-leucine (Sigma) as a standard in the range from 0.25 to 2 mmol L^{-1}.

2.10. Enzyme linked immunosorbent assay (ELISA) method

Indirect ELISA was used to determine the serum IgG and IgA End Point Titer (EPT). The microtitre plate (MaxiSorp NUNC) was coated with 50 μL/well of pure antigen, diluted in 10 mM PBS, pH 7.4, and incubated for 12–18 h at 4 °C. Residual free binding sites were blocked with 1.5% BSA (Sigma) in PBS for 1 h at 37 °C. The plate was then washed four times with PBS containing 0.5% Tween 20. This washing system was used after each analytical step. Next, the mice serum samples were added. The plate was then incubated for 1 h at 37 °C. After washing, the plate was incubated for 1 h at 37 °C with peroxidase-conjugated goat anti-mice immunoglobulin G or A (Sigma, adequately A4416 or A4789), followed by washing and addition of ABST (Moss) substrate. After incubation for 30–60 min, absorbance was read at 492 nm on an automatic plate reader (JUPITER UVM-340, Biogenet). The EPT was expressed as logarithm of base 2 from dilution. The analysis was performed in duplicate.

2.11. Lymphocyte isolation

Lymphocytes from spleen (SPL) and mesenteric lymph (MLN) nodes were isolated according to standard method (Kruisbeek, 2000). MLN mononuclear cells suspension was obtained using dounce homogenization and centrifugation at 1500*g*. SPL mononu-

clear cells suspension was subjected to Ficoll type 400 (Sigma) density gradient centrifugation.

Cell suspensions were cultured in medium and supplemented with TA and GTA at 37 °C in atmosphere of 5% CO_2. RPMI 1640 medium was purchased from Sigma and supplemented (v/v) with 10% heat inactivated fetal bovine serum (GIBCO), 1% non-essential amino acids (Sigma, M7145) and 1% penicilin-streptomicin solution (Sigma, G6784).

2.12. B cell Ab ELISPOT

For Ab-formatting cells (AFC) counting IgA and IgG antigen specific ELISPOT was done according to standard method (Maddaloni et al., 2006). Mixed cellulose ester membrane-bottomed microtiter plate (Millipore) were coated with 100 μg/mL of antigen in sterile PBS overnight at room temperature. The plate was blocked with complete medium at 37 °C for 1 h, then plate was washed four times with PBS containing 0.5% Tween 20. As a next step 100 μL of varying concentration of cell suspension was added to well and plate was incubated overnight at 37 °C in 5% CO_2 atmosphere. For detection AFC response anti-mouse IgG and IgA-HRP conjugates (Sigma) were added. Then plates were incubated in humidity chamber overnight at 4 °C. After washing plates were developed with AEC (Moss) substrate (~1 h). The reaction was stopped with H_2O. On the next day AFC were counting under stereo zoom microscope (OLYMPUS, SZX 9).

3. Results and discussion

3.1. Glycation progress

The content of protein in lyophilized pea albumin powder was determined and accounted for 0.38 mg protein/mg^{-1} lyophilisate. Molecular weight profiles before and after glycation were determined by size exclusion chromatography (Fig. 1) and SDS–PAGE electrophoresis (Fig. 2). Both TA and GTA were characterized by a similar elution pattern from size exclusion chromatography column. There were observed three peaks on chromatograms corresponding to molecular weights of approx. 66 kDa, 20 kDa and 9 kDa. It was found by means of SDS–PAGE that total pea albumins extract was characterized by proteins fractions with molecular weights in the range from 66.0 to 6.5 kDa. The protein bands

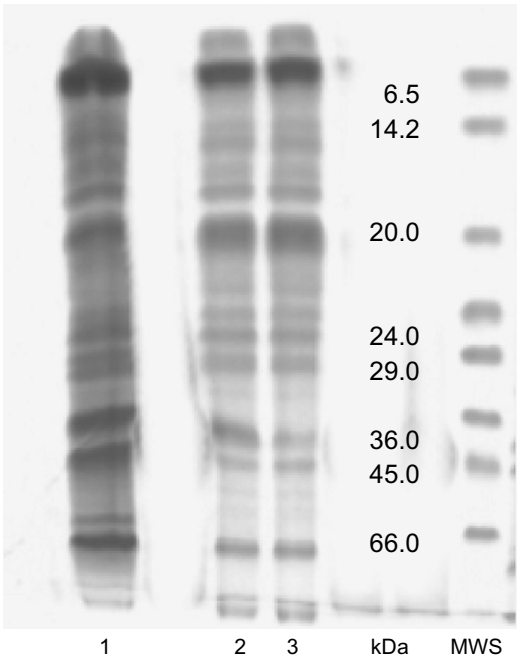

Fig. 2. SDS–PAGE of total pea albumins (line 1) and glycated total pea albumins (lines 2 and 3).

detected in TA were in the same molecular size range in our experiment as it has been reported by other authors (Croy, Hoque, Gatehouse, & Boulter, 1984; Urbano et al., 2005). Two intensively stained albumin fractions on electropherograms were of molecular weights of about 6.5 kDa and 20 kDa, and it can be concluded that they can, respectively, correspond to PA1 and PA2 found in green pea by Jouvensal et al. (2003), Wang et al. (2003), and Bérot, Le Goff, Foucault, and Quillien (2007). The glycated total pea albumins were characterized by similar molecular weight distribution as compared to total pea albumins, however when the protein bands were stained with Glyco gel Code dye for glycated proteins it was observed on electropherogram (Fig. 3) that glycoproteins in TA gave one intense purple-pink band with molecular weight of about 6.5 kDa and samples after glycation had two stained glycoprotein

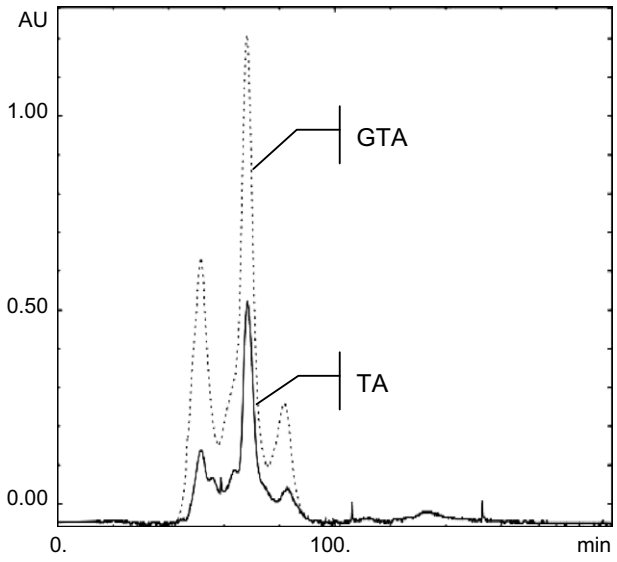

Fig. 1. Size exclusion chromatography of total pea albumins (TA) and glycated total pea albumins (GTA).

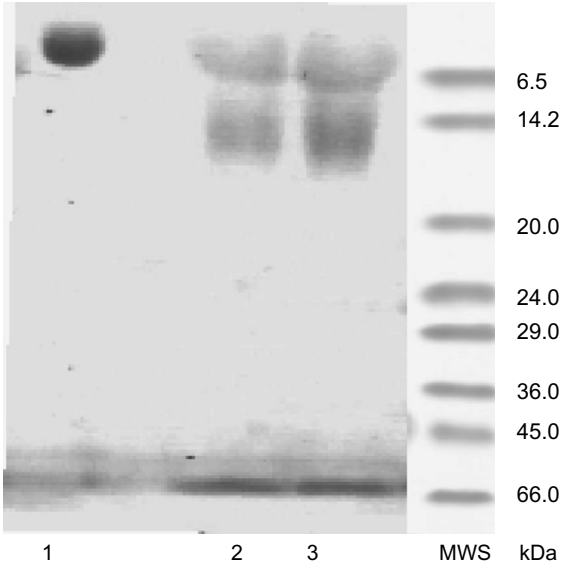

Fig. 3. SDS–PAGE stained with GelCode Glycoprotein of total pea albumins (line1), glycated total pea albumins (lines 2 and 3).

bands of molecular weights of about 6.5 and 20 kDa. The results obtained proved that some glucose bound to pea albumin during the 7 day process.

Progress in protein glycation was also confirmed by free amino acid groups determination. This factor decreased in the course of the experiment, and prior to glycation accounted for 0.07 ± 0.003 and after glycation for 0.06 ± 0.0026 mgLeu/mg sample. It was found that after 7 days of glycation about 15% of free amino acid residues were blocked by glucose.

Reassuming the presented results it can be suggested that the total pea albumin includes proteins of molecular masses in the range from 6 to 66 kDa with two highly predominant fractions of molecular masses of ~6.5 kDa and ~20 kDa which correspond to two main pea albumin fractions PA1 and PA2 observed by other authors. A conclusion can be drawn that size exclusion chromatography and SDS–PAGE electrophoresis are not sufficient techniques to determine the progress of glycation.

3.2. Animal experiment

Mice were orally immunized on 0, 1, 2, 10 day *vs.* 0, 7, 14 day with TA. Serum and fecal samples were collected on 28, 42 and 56 day post immunization and then serum IgG and IgA end point titer as well fecal IgA against TA were determined. Serum IgG EPT level was observed to be about 2^7 and there were no significant differences found between these two groups (Fig. 4). Serum and fecal IgA end point titer was below detectable level (results not shown). On the basis of results obtained 0, 7 and 14 day immunization was selected to be performed in the further experiment.

No significant differences were observed in IgG titers during oral immunization of mice (on 0, 7 and 14 day) with TA *vs.* GTA (Figs. 4 and 5). IgA level was undetectable in serum and fecal samples (not shown). It confirms our hypothesis that glycated green pea albumins administered orally do not induce the mucosal immune system response.

Changing the route of immunization altered the immune system response to a high extent. A group of five mice was immunized

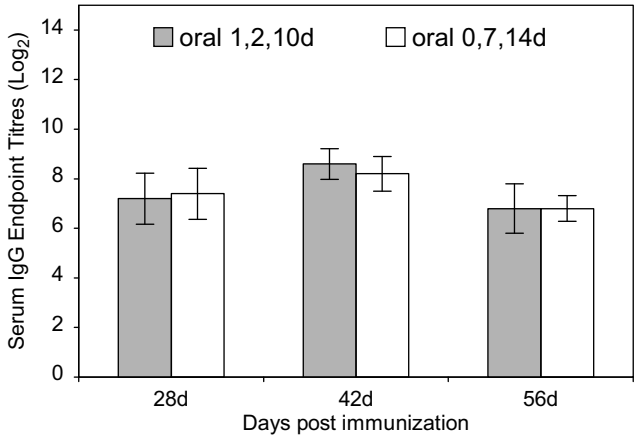

Fig. 4. Serum IgG titer after oral mice immunization with total pea albumins on day 0, 1, 2, 10 and 0, 7, 14.

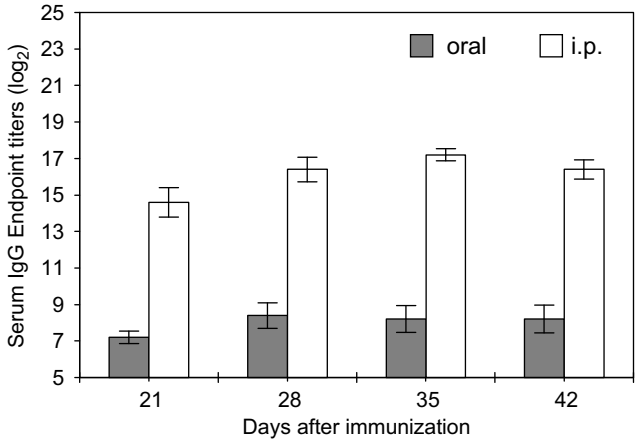

Fig. 5. Serum IgG titer after oral and intraperitonel mice immunization with glycated total pea albumins on day 0, 7, 14.

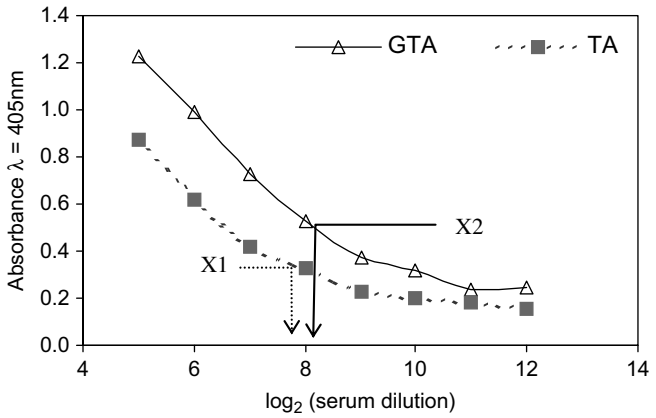

Fig. 6. Cross reactivity of anti-total pea albumins antibodies with glycated pea albumins. X1 value for 50% antibodies anti-TA binding with TA, X2 value for 50% antibodies anti-TA binding with GTA.

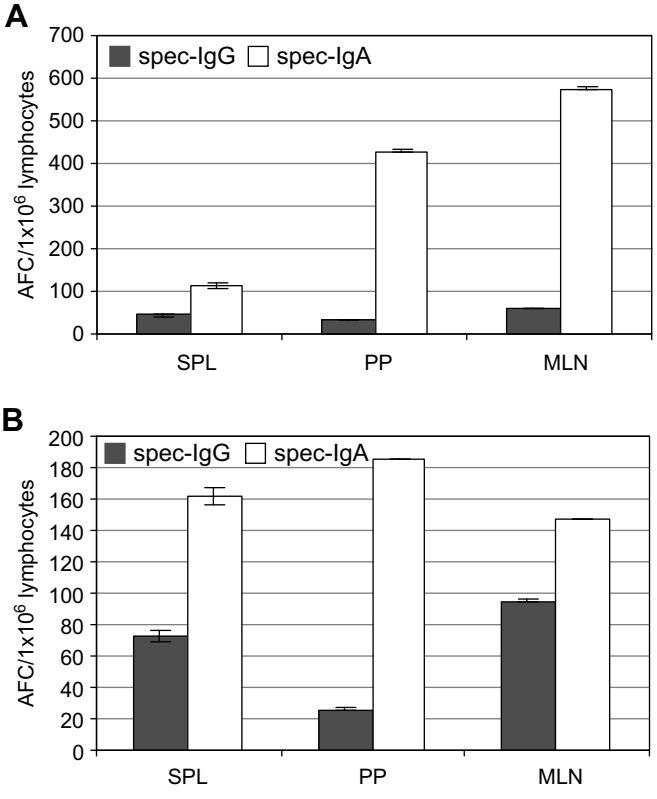

Fig. 7. The B-cells ELISPOT for specific IgG and IgA. (A) SPL, PP, MLN cells of mice orally immunized with TA. (B) SPL, PP, MLN cells of mice orally immunized with GTA.

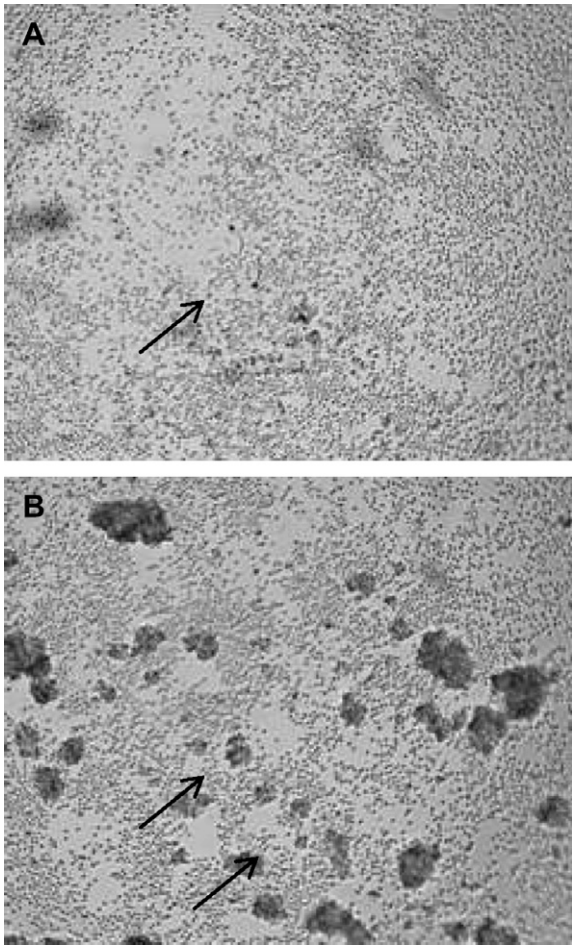

Fig. 8. Culture of SPL lymphocytes from orally immunized mice with TA, without antigen stimulation (A) and with antigen stimulation (B).

intraperitoneally with GTA on day 0, 7 and 14 *vs*. orally (Fig. 5). Serum IgG level was much higher for i.p. route as compared to the oral route. It is clear that i.p. immunization resulted in about twofold higher IgG response as compared to the oral immunization and specific serum IgG accounted for $2^{14} \pm 0.81$; $2^{16} \pm 0.67$; $2^{17} \pm 0.33$; $2^{16} \pm 0.53$, respectively, on 21, 28, 35, 42 day post immunization.

Cross reactivity of antibodies produced against glycated green pea proteins was examined by ELISA (Fig. 6). It was found that the antibodies produced cross reacted with total pea albumins at a high level of 92%. It has definitely confirmed that glycated albumins have epitopes very similar to those present in the non glycated (TA) form. It is suggested that glycation does not decreases pea albumins immunoreactivity.

An antigen specific B cell ELISPOT assay confirmed ELISA results at cellular level (Fig. 7). There are no significant differences in response irrespective whether TA or GTA were used for the oral immunization. The i.p. immunization only increased number of antigen formatting cells (AFC) for SPL specific IgG.

Spleen lymphocytes were cultured in medium (Fig. 8A) and with stimulation with antigen (Fig. 8B). When antigen was added to the medium, the growth of lymphocytes was stimulated and an increased number of proliferating cells was observed.

4. Conclusion

The conclusion is that glycation does not change the immunoreactivity of proteins to a high extent and during the presented route of immunization TA and GTA could induce specific tolerance mechanism.

Pea albumins before and after glycation do not induce mucosal immune system under experimental conditions, which is very important from the nutritional point of view.

Culture stimulation with antigen increases cells proliferation and suggests that some of lymphocytes were sensitized with investigated proteins during the experiment. The route, doses and time of antigen administration, can independently induce tolerance by a few mechanisms and, according to literature, suppression is considered to be possible (Fujihashi & McGhee, 2004).

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