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EFFECT OF MODIFICATIONS OF PEA ALBUMINS ON THEIR IMMUNOREACTIVE PROPERTIES

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The aim of this study was to investigate the effect of non-enzymatic glycosylation and hydrolysis of pea albumins with pepsin on their immunoreactive properties. Albumin fraction was isolated from pea seeds an then glycated and hydrolysed by pepsin. Pea albumin was characterised by SDS--PAGE and glycotest. A 15% progress in non-enzymatic glycosylation was found. The *in vivo* experiment demonstrated the influence of glycation on mouse mucosal immune system. The influence of native, glycated and hydrolysed pea albumins on spleen (SPL) and mesenteric lymph nodes (MLN) lymphocytes proliferation was investigated. The culture MLN lymphocytes showed an increase in proliferation during stimulation with all of antigens (native, glycated, hydrolyzed). The proliferation was higher in MLN lymphocyte of the intraperitoneally-sensitized group. This observation suggests that the route of immunization can affect their immunoreactivity. SPL lymphocytes of the orally-immunized group showed higher proliferation as compared with SPL lymphocytes of the group sensitized before oral immunization. It is likely that the route of antigen administration has induced a specific food tolerance. The results suggest that none of the modifications performed has changed the immunoreactivity of the investigated proteins to a great extent.

INTRODUCTION

Plant seeds are essential for human and animal nutrition [Sathe *et al*., 2005; De Graaf *et al.,* 2001]. Pea (*Pisum sativum*) is one of them. Pea seeds are characterised by a high content of protein, dietary fiber, minerals, vitamins and antioxidant compounds [Alonso *et al.*, 2001; Urbano *et al.,* 2005]. Pea proteins are of very high nutritional value due to amino acid composition, as they contain a lot of sulphur amino acids. The seeds of pea contain two major classes of proteins: albumins – about 30% of total proteins – and globulins. Two main fractions are observed within albumin fraction: PA1, 4.5% of the total pea seeds protein, specially enriched in sulphur amino acids constitute ~50% of the total sulfur amino acids in the pea [Higgins, 1986] and PA2, which contribute about 16% of the total sulfur amino acids [Lu *et al*., 2000]. PA1 and PA2 are well characterised regarding amino acids sequences, molecular weights, structures, cDNA and gene identification [Higgins *et al.*, 1986*,* 1987]. Pea seed proteins can induce food intolerance or food hypersensitivity [Breitender & Radauer, 2004; Sampson, 2003].

It is estimated that about 6% of the infants population suffer from food allergy [Sampson, 2003] and statistically 1-3% of the adult population [Sell *et al*., 2005; Zeiger, 1990]. Food allergy symptoms include urticaria, allergic asthma, nausea, vomiting *etc*. and sometimes may result in death [Sathe *et al.,* 2005; Sampson, 2003]. In the European countries *ca*. 3.3% of allergic population have allergy developed to beans and 0.8% to green pea [Sell *et al*., 2005]. Allergy to pea proteins has not been intensively investigated and pea allergens have been characterised to a lesser extent as compared to others, thus it appears to be an interesting area for research. Typical pea allergy is contributed to pea globulin fractions Pis s1 (vicilin) and Pis s2 (convicilin) [Sathe *et al.,* 2005; Sanchez-Monge *et al*., 2004]. Malley *et al*. [1975] determined molecular mass of green pea allergen as 11 kDa and showed that only albumins gave positive results in a skin test. It is a new hypothesis in allergy to pea that two albumin fractions PA1 and PA2 may be more allergenic than globulins. Vioque *et al*. [1998] reported cytosolic albumin (PA2), which is not hydrolysed during germination, to be responsible for allergic reaction in chickpea-sensitive individuals. Croy *et al*. [1984] described pea albumins as the main nutritional components. Sell *et al*. [2005] and Wensing *et al*. [2003] considered them responsible for allergy cause.

The aim of our study was to determine the effect of glycation and hydrolysis of pea albumins on their immunoreactive properties. Glycation can occur spontaneously during a number of technological treatments applied in industrial food processing, and is officially referred to as the Maillard reaction. It is based on binding a sugar molecule to protein. End products are characterised by different physical and chemical properties and different immunoreactivity. Pea albumins were extracted from flour and purified, subsequently the process of glycation or hydrolysis was performed.

Total pea albumins (TA) and glycated pea albumins (GTA) were orally and intraperitoneally administrated to Balb/C mice. Tissue culture experiments were conducted to

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analyse 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.

MATERIALS AND METHODS

Materials and sample preparation

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

Extraction of total albumin

Pea flour (50 g) was extracted with 1000 mL of H_2O d.d. at 4°C for 24 h, then the extract was centrifuged (22,500×g, 4°C, 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 total pea albumin powder was further referred to as the total pea albumins [Higgins *et al.,* 1986].

Non-enzymatic glycosylation of proteins

Glycation was performed as previously described [Cazacu-Davidescu *et al.,* 2005] with some modification of mixture content, *i.e*. 0.380 mg of TA and 0.760 g of D(+) glucose (Sigma) were reconstituted in 20 mL sterile PBS (containing streptomycin, penicillin and gentamycin). The bottle was closed in sterile and left at 37°C for 7 days. Then, the solution was dialyzed against distilled water and concentrated with polyethylene glycol. Aliquots of glycated total pea albumins were frozen at -20°C for other experiments.

Hydrolysis of total pea albumin

An extract of pea albumins (5%) was prepared in 0.06 mol/L HCl and heated up to 37°C to obtain the final volume of 10 mL. Aliquots of pepsin (EC 3.4.23.1; activity 452 U/mg solid; Sigma) solution (2 mg/mL in 0.01 mol/L HCl) were added at the ratio of 1:5. Hydrolysis was carried out with 0.1 mol/L HCl at constant pH 2.0, at 37°C for 1.5 h [Bhatty & Patel, 1983]. The solution of pea albumins and pepsin as control was incubated in the same conditions. The enzymatic reaction was stopped with 1 mol/L NaOH at the final pH 7.0. The analysis was performed in duplicate.

Mice experiment

Balb/C mice, 6- to 8-week old, were purchased from the Medical Research Center PAS, Warsaw. Mice of group 1, orally immunized on day 0, 7, 14 with 250 μL of PBS, were used as a control group. Mice of group 2 and 3 were immunized orally, respectively GTA and TA, on days 0, 7, 14. Mice of group 4 and 5 on day -3 were intraperitoneally sensitized with 100 μg of TA in the precence of Complete Freud Adjuvant and on day 0, 7, 14 were orally immunized with 2 mg of TA (group 5) or GTA (group 4) in 250 μL of PBS. Oral doses were administered with a 21-gauge gavage needle attached to a 1 mL tuberculin syringe directly to the stomach.

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

SDS-PAGE

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 BIO-RAD electrophoretic equipment. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Sigma) and Glycoprotein Detection Kit (Sigma). Low molecular weight standards (LMWS) in the range from 6.5 kDa to 66.0 kDa (Sigma) were used for mass determination.

Protein bound sugar assay

The quantity of protein bound sugar was determined using the periodate method [Ahmed & Furth, 1991]. A sample (40 μL) was incubated with 20 μL of 0.1 mol/L HCl and 20 μL of 0.05 mol/L $NaIO_4$ at room temperature for 30 min. Then the samples were cooled in ice for 10 min and mixed with 20 μL of 15% $ZnSO_4$ and 0.7 mol/L NaOH. The mixture was centrifuged for 10 min at 9000×g and 100 μL of supernatant was transferred to microplate. Then 200 μL of freshly prepared reagent (46 μL of acetylacetone in 10 mL of 3.3 mol/L ammonium acetate) were added to each well. The plate was mixed and incubated for 1 h at 37°C. The absorbance against reagent blank was read at λ=405 nm on an automatic plate reader (Jupiter UVM-340, Biogenet). A calibration curve was obtained by using D(+)glucose (Sigma) as a standard in the range from 0 to 1000 nmol/mL. The analysis was performed in triplicate.

Lymphocyte isolation

Lymphocytes from spleen and mesenteric lymph nodes were isolated according to a standard method [Maddaloni *et al*., 2006]. Cells at a concentration of 2×10^5 cell per 100 μL were cultured in RPMI 1640 medium (Sigma) supplemented with 10% heat inactivated fetal bovine serum (FBS, GIBCO), 1% non-essential amino acids (Sigma) and 1% penicilin-streptomicin solution (Sigma), at 37°C in the atmosphere of 5% CO_2. Lymphocytes were cultured in medium only and in presence of TA, GTA or hydrolyzed TA (HTA).

Determination of viable cells

Cells were centrifuged and resuspended in HBSS. The cell suspension (10 μL) was mixed wit 40 μL of 0.4% Trypan Blue solution [Klein *et al*., 2006]. Live cells were counted on a hemacytometer (Sigma). Analysis was performed in duplicate.

RESULTS AND DISCUSSION

Glycation progress

Molecular weight profile of total albumin extract was determined by SDS-PAGE (Figure 1, line TA). The protein bands found by means of SDS-PAGE showed that total pea albumins extract was characterised by protein fractions of molecular weights in the range from 66.0 to 6.5 kDa. The same results have been reported by Urbano *et al.* [2005] and Croy *et al.* [1984]. Two intensively stained albumin fractions on electro-

phoregrams were of molecular weights of about 6.5 kDa and 26 kDa. They can, respectively, correspond to PA1 and PA2 found in green pea by Jouvensal *et al*. [2003] and Wang *et al*. [2003]. The result of SDS-PAGE of glycated total pea albumins (Figure 1, line GTA) shows similar molecular weight distribution as compared to total pea albumins. The protein bands before and after glycation were stained with Glycotest for glycated proteins (Figure 2). Glycoproteins in TA gave week purple-pink band with molecular weight of about 6.5 kDa and samples after glycation exhibited the band with the same r_f factor but more intense colour (OD checked was higher). On GTA line a week band was observed around molecular weight 26 kDa. The content of protein in separated samples were the same, which suggests that results obtained proved that some glucose has bounded to pea albumin during the 7-day process.

The progress in protein glycation was also confirmed by glucose bounded assay (Figure 3). It was calculated that 15% more glucose was bound to total pea albumins after spontaneous 7-day glycation. On the basis of the results obtained it can be concluded that glycation occurs mostly within two main albumin fractions: PA1 and PA2.

Total pea albumins was hydrolysed by pepsin (HTA). The SDS-PAGE was performed to characterize molecular weight distribution after the process of hydrolysis (Figure 4). It was found that pepsin hydrolysis changed molecular weight distribution within TA to a high extent. Lines HTA in Figure 4 showed week bands in the range 45-66 kDa, which could correspond to pepsin separation (Figure 4, line P). It looks like most proteins were digested by pepsin and gave broadening band starting at about 15 kDa.

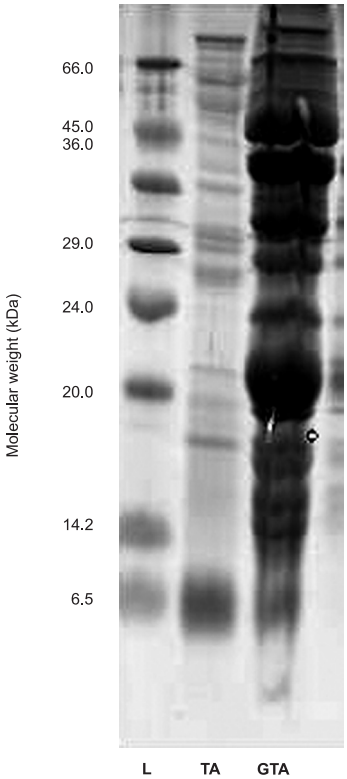

FIGURE 1. SDS-PAGE separation of native (TA) and glycated (GTA) pea albumins. L – low molecular weight marker.

Animal experiment

Mice were orally immunized on day 0, 7 and 14 with TA and GTA. Groups 4 and 5 were sensitized three days before with emulsion containing 100 μg of TA and 100 μL of Complete Freud Adjuvant.

On day 49 the mice were killed and B-lymphocytes were isolated from spleen (SPL) and mesenteric lymph nodes (MLN). The mononuclear cells (2×10^5/well) were cultured for 48 and 72 h in a 96 bottom plate. After 12 h, lymphocytes were stimulated with TA, glycated TA and hydrolyzed TA. After 2 and 3 days, growing cells number were calculated using the trypan blue method.

SPL lymphocytes from mice treated with PBS did not show growing activity during antigen stimulation (data not

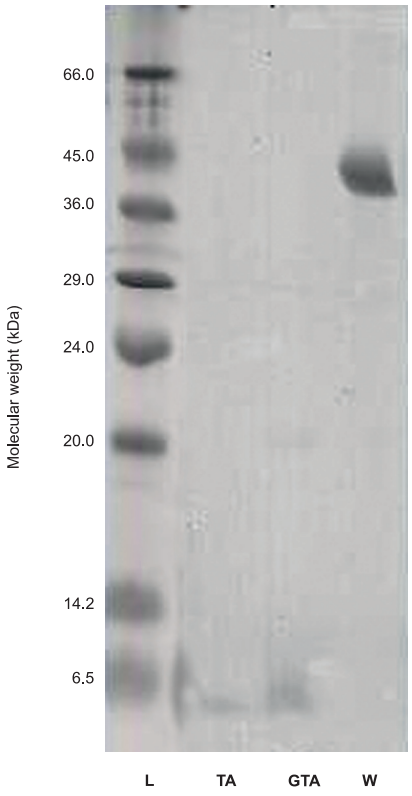

FIGURE 2. SDS-PAGE separation of native (TA) and glycated (GTA) pea albumins, stained for glycoproteins. L – low molecular weight marker; W – glycoprotein marker.

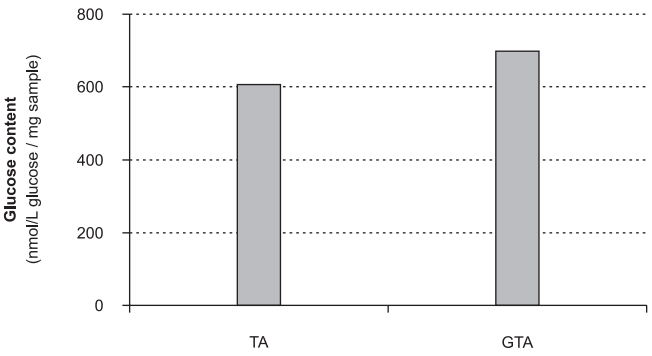

FIGURE 3. The content of glucose bound to proteins before (TA) and after (GTA) glycation.

shown). SPL lymphocytes from mice treated orally with TA showed an increase in cells number after 2 days of stimulation with TA (Figure 5A). The culture stimulated with GTA showed similar proliferation as compared to that cultured in medium only. SPL lymphocytes from mice treated with GTA (group 3) showed less intensive growth of cells as compared to lymphocytes from group 2 (Figure 5B).

SPL lymphocytes isolated from sensitized groups number 4 and 5 (Figure 5 C,D) showed week growth activity. Any antigen stimulation did not induce an increase in cell counts in comparison with medium.

A better response was observed in MLN lymphocytes culture, which may be attributed to the route of immunization (Figure 6 A-D). Lymphocytes of all groups showed higher proliferation activity as compared to lymphocytes isolated from the control group. In each case, the highest results were obtained for lymphocytes of group 5 – sensitized with TA and orally immunized with GTA (Figure 6 A-D, column 5). In summary, the results obtained suggest that experimental route of immunization poorly induces mucosal immunity of mice. MLN B-lymphocytes – inductive sites of GALT – were activated and the observed weak response of SPL lymphocytes could suggest that tolerance mechanism could be activated.

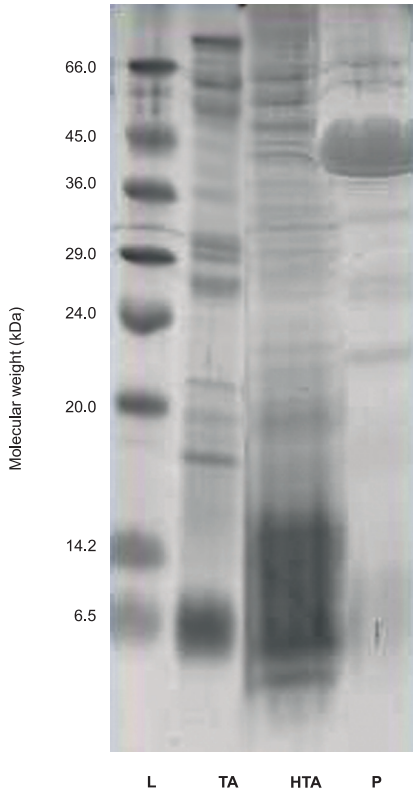

FIGURE 4. SDS-PAGE separation of native (TA) and hydrolyzed (HTA) pea albumins. L – low molecular weight marker; P – pepsin.

CONCLUSIONS

As results have shown, SDS-PAGE method is not sufficient to determine the progress of non-enzymatic glycation. Using colorimetric method for determination of glucose bound protein, 15% glycation was found for the the experimental 7-day process. It was shown that glycation occurred within allergenic fractions PA1 and PA2.

Culture stimulation with antigen increased the proliferation of MLN cells, which suggests that some of lymphocytes have been sensitized with the 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

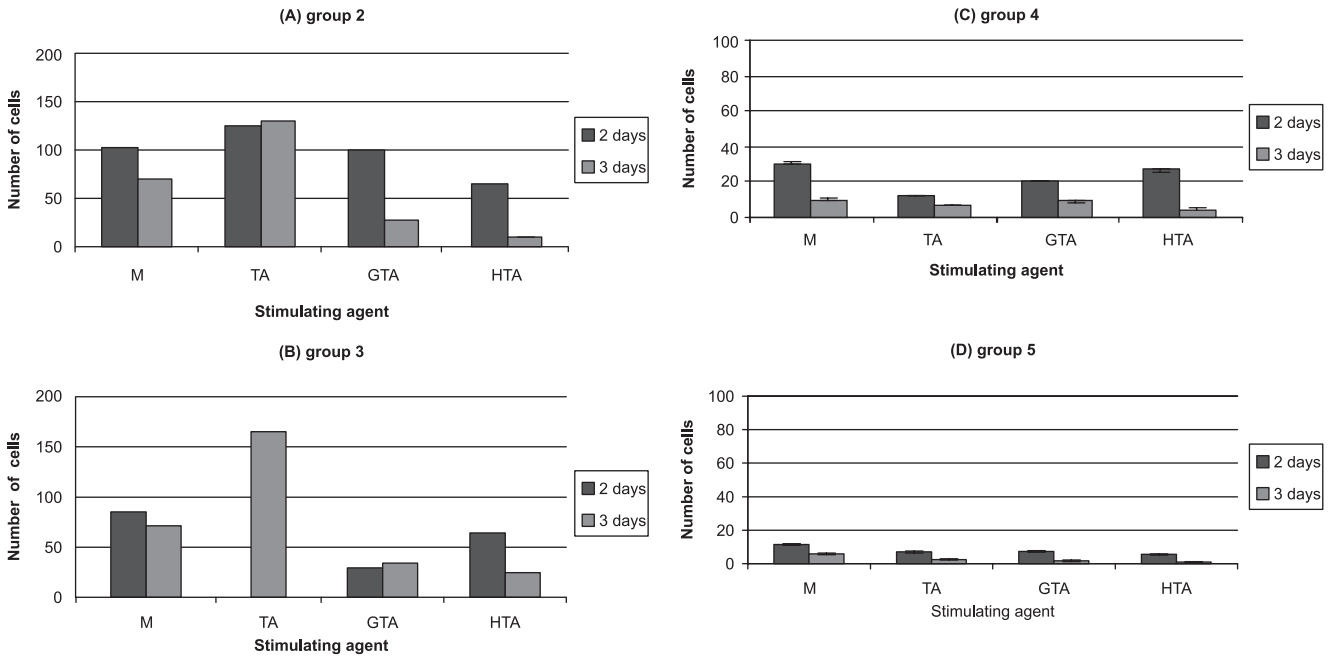

FIGURE 5. Effect of addition of total albumin (TA), glycated TA and hydrolyzed TA on spleen lymphocytes viability in mice of group 2 (A) – mice orally immunized with glycated TA, group 3 (B) – mice orally immunized with TA, group 4 (C) – mice sensitized and orally immunized with glycated TA, group 5 (D) – mice sensitized and orally immunized with TA.

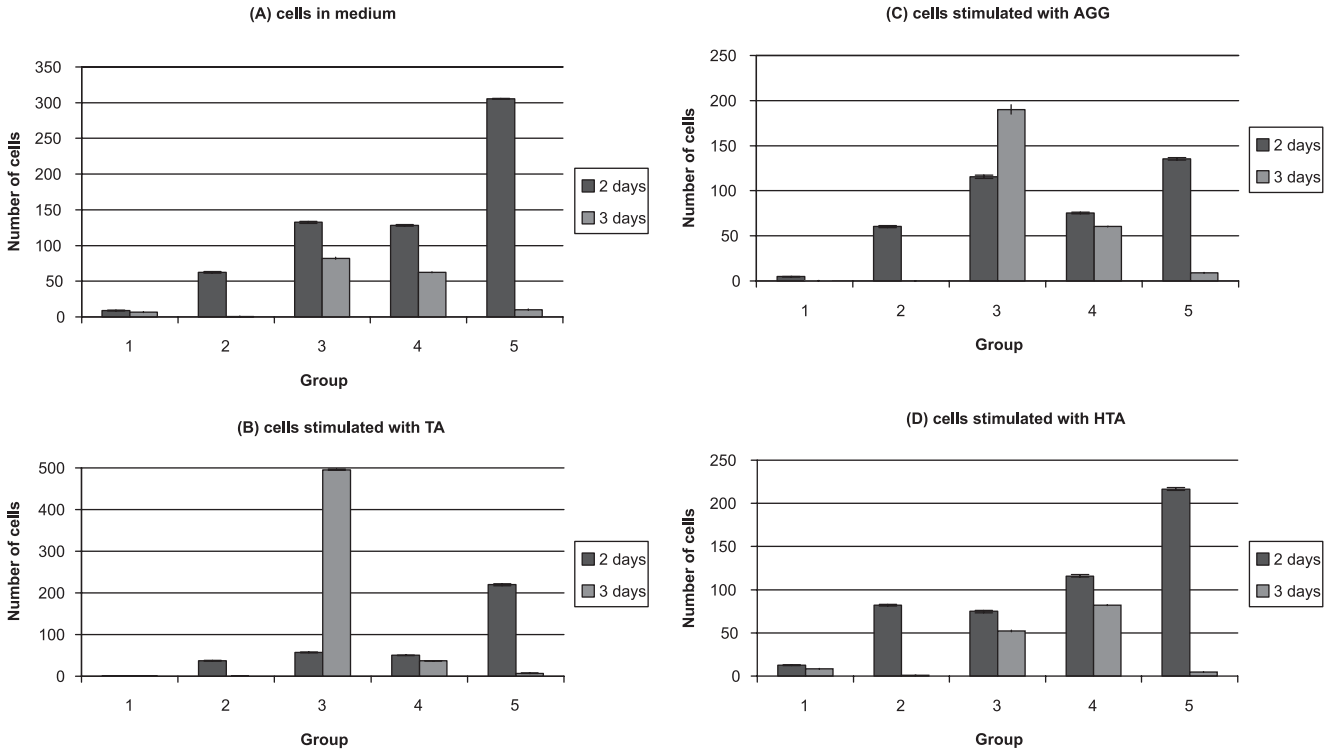

FIGURE 6. Viability of lymphocytes from mesenteric lymph nodes in medium (A), in medium with TA (B), in medium with glycated TA (C) and in medium with hydrolyzed TA (D). Cells were isolated from mice orally immunized with PBS (group 1), TA (group 2) and with glycated TA (group 3) and sensitized and orally immunized with glycated TA (group 4) and TA (group 5).

possible [Fujihashi & McGhee, 2004].

The 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. The presented antigen immunization does not induce mucosal immune system under experimental conditions (without mucosal adiuvant), which is very important from the nutritional point of view.

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