RESEARCH ARTICLE

Sensitizing potential of enzymatically cross-linked peanut proteins in a mouse model of peanut allergy

Jelena Radosavljevic[1], Emilia Nordlund[2], Luka Mihajlovic[1], Maja Krstic[1], Torsten Bohn[3], Johanna Buchert[2], Tanja Cirkovic Velickovic[1] and Joost Smit[4]

[1] University of Belgrade, Faculty of Chemistry, Belgrade, Serbia
[2] VTT Technical Research Centre of Finland, Espoo, Finland
[3] Centre de Recherche Public – Gabriel Lippmann, Belvaux, Luxembourg
[4] Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands

Received: June 3, 2013
Revised: July 22, 2013
Accepted: July 30, 2013

Scope: The cross-linking of proteins by enzymes to form high-molecular-weight protein, aggregates can be used to tailor the technological or physiological functionality of food products. Aggregation of dietary proteins by food processing may promote allergic sensitization, but the effects of enzymatic cross-linking of dietary proteins on the allergenic potential of food are not known. In this study, the bioavailability and the sensitizing or tolerizing potential of peanut proteins (PE) cross-linked with microbial tyrosinase from *Trichoderma reesei* and mushroom tyrosinase from *Agaricus bisporus*, were investigated.

Methods and results: The impact of cross-linking of PE on the in vitro bioavailability of fluorescein isothiocyanate-labeled peanut proteins was tested in a Caco-2 cell monolayer and by competitive ELISA. The in vivo allergenicity or capacity to induce oral tolerance in mice were measured by serum levels of PE-specific antibodies and T cell cytokine production after exposure to PE and cross-linked PE.

Conclusion: Enzymatic processing of peanut proteins by the two tyrosinases increased the bioavailability of major peanut allergen Ara h 2, but did not significantly change the allergenic or tolerizing properties of peanut. Enzymatic treatment of peanut proteins yielded cross-linked proteins with preserved molecular and immunological features of peanut allergens.

Keywords:
Allergenicity / Protein cross-linking / Peanut / Tyrosinase

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1 Introduction

The increase in diet-related disorders including food allergies in industrialized countries has been linked to the changes in nutritional habits and consumption of increasingly processed food [1]. Food processing may change the intrinsic allergenicity of proteins. However, until now, no specific variables are available that could be used to reliably determine how processing will influence protein allergenicity [2]. The effects of food processing on the allergenic potential of proteins and foods may involve both the sensitization and effector phases of food allergy by influencing allergen stability and conformation, digestibility in the gastrointestinal (GI) tract, route of uptake through the mucosa and aggregation of processed food allergens [2, 3].

For instance, it has been shown that heating, pasteurization, and other methods of food processing have different effects on food allergens, even when contained in the same complex food matrix [1, 4, 5]. Heating generally decreases protein allergenicity by destroying conformational epitopes [6]. In contrast, in peanut and shrimp, heat-induced glycation by the Maillard reaction may increase allergenicity [1], while

Correspondence: Professor Tanja Cirkovic Velickovic, University of Belgrade, Faculty of Chemistry, Studentski trg 16, 11 000 Belgrade, Serbia
E-mail: tcirkov@chem.bg.ac.rs

Abbreviations: GI, gastrointestinal; **L-DOPA**, 3,4-dihydroxy-L-phenylalanine; **mMCP-1**, mouse mast-cell protease-1; **PE**, peanut proteins; **SEM**, scanning electronic microscopy; **TA**, peanut extract cross-linked with tyrosinase from *A. bisporus*; **TT**, peanut extract cross-linked with tyrosinase from *T. reesei*

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sonication induced structural changes in whey allergens did not affect the allergenicity of the proteins [7].

The increased food processing in order to provide food with enhanced organoleptic properties or functions also bears the risk of increasing allergenicity of dietary proteins [4]. Several factors are known to be of importance for the induction of an allergenic response to dietary proteins. These can be divided into intrinsic protein factors and host factors. The level of aggregation of dietary proteins is considered to be the most important intrinsic factor for the development of an allergic response. Highly aggregated, cross-linked, and/or thermally processed food proteins show a different behavior in the digestive fluids, providing a rationale for the possible persistence of intact proteins and larger peptides in the GI tract [8]. On the other hand, intrinsic stability of proteins-to-pepsin digestion and/or impairment of gastric digestion due to antiacid medications intake have been shown to contribute to the allergic response development in animal models [9]. Thus, food allergen digestion, aggregation, and route of uptake in the GI tract are considered to be of major importance for the allergenic propensity of food [10–12].

Cross-linking enzymes are currently used to tailor food functionality [5, 13]. Cross-linking of dietary proteins can increase the molecular weight of proteins, change its 3D structure, charge, or surface characteristics of the molecule. Enzymatic protein cross-linking can also alter the biological properties of food allergens, including susceptibility to proteolysis by digestive enzymes, solubility, ligand binding, IgE binding, and antigenicity [8, 14–16]. Various oxidase enzymes, such as tyrosinase (EC 1.14.18.1) from mushroom *Agaricus bisporus* and tyrosinase from filamentous fungi *Trichoderma reesei*, have been investigated as novel cross-linking tools in food processing [17]. Oxidases have potential in food structure engineering as they can catalyze cross-linking between proteins, between polysaccharides, and between proteins and polysaccharides [13]. Tyrosinase acts on proteins and peptides by catalyzing oxidation of tyrosine and making oxidative cross-linking of tyrosine side-chains [17, 18].

Peanut allergy is the most prevalent food allergy, with an estimated 1–2% of the total children population showing allergic symptoms [19, 20]. It is also a frequent cause of anaphylactic reactions and deaths in children and adults [21–23]. The propensity of peanut allergens to aggregate, also after enzymatic digestion, has been associated with their high potential to induce allergic sensitization [10]. Therefore, since peanut is such a potent cause of food allergies, enzymatic treatment and formation of stable covalent cross-linked aggregates of peanut allergens might represent an additional risk factor for development of food allergies that has not been tested previously.

In this study we applied *A. bisporus* and *T. reesei* tyrosinases to cross-link peanut proteins and test effects of protein covalent cross-linking into high-molecular-weight aggregates on peanut allergy development and oral tolerance induction in vivo.

2 Material and methods

2.1 Chemicals and standards

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade or superior.

2.2 Preparation of peanut extracts

Peanut proteins were extracted according to Radosavljevic et al. [24] with slight modifications: prior to extraction, raw peanuts were defatted with petrol-ether and 50 mM ammonium bicarbonate was used during the extraction procedure.

2.3 Cross-linking reaction

For cross-linking of peanut extract tyrosinases from *T. reesei* (yielding cross-linked PE marked as TT) and *A. bisporus* (yielding cross-linked PE marked as TA) were used.

Tyrosinase from *T. reesei* was purified and characterized as described previously [25, 26]. Tyrosinase from *A. bisporus* was purchased from Fluka/Sigma (Seelze, Germany). Enzymatic activity of tyrosinases (1900 nkat/mL for *T. reesei* and 320 nkat/mL for *A. bisporus*) were measured using 3,4-dihydroxy-L-phenylalanine as a substrate [25]. Experimental conditions used for cross-linking of peanut proteins (PE) were preliminary tested in order to provide the highest degree of protein cross-linking (Supporting Information Figs. 1 and 2).

First, the PE lyophilisate was dissolved in 100 mM phosphate buffer pH 7.0 for production of TA, or 100 mM phosphate buffer pH 8.0 for production of TT. Peanut protein (PE) concentration was adjusted to 5 mg/mL. Enzymes were used at 1000 nkat/g PE according to the measured enzyme activities using 3,4-dihydroxy-L-phenylalanine as substrate. Cross-linking was performed for 24 h at 37°C with mixing after which the obtained material was extensively dialyzed against ammonium-bicarbonate buffer (50 mM and pH 8.0) and lyophilized.

2.4 Scanning electronic microscopy and electrophoretic analysis of the obtained material

Solutions containing 1 mg/mL of protein in PBS as determined by the BCA assay were air-dried and gold-covered by cathodic spraying (LEICA SCD005, Leica Microsystems, Germany). Morphology of the materials was analyzed by a scanning electronic microscope (JSM-6610LV, Jeol, Peabody, MA, USA). The scanning electronic microscopy (SEM) observation was done under the following conditions: magnification of 20 000×, 30 kV, WD = 7 and Signal A = SE_I.

SDS-PAGE on 8 and 12% gels was performed according to Laemmli [27] and stained with Coomassie Brilliant

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Blue R250 (Serva Electrophoresis GmbH, Heidelberg, Germany). For comparing higher molecular masses of samples, electrophoresis on 1% agarose gels was performed, with custom molecular markers made from mouse myofibrilar proteins [28]. In all electrophoretic analyses 40 μg of proteins (determined by BCA assay) were loaded per lane.

2.5 Inhibition ELISA with anti Ara h antibodies

Inhibition ELISAs with polyclonal rabbit sera against individual allergens were performed as previously described, with some modifications [29]. Briefly, plates were coated overnight with PE, and blocked with 1% BSA in 0.1% Tween-TBS for 2 h. Peanut cross-links were prepared at tenfold serial dilutions (range from 1–10^{-7} mg/mL (dry weight/volume)) and incubated for 1 h with antibodies against Ara h 1, Ara h 2, Ara h 3, and Ara h 6 at room temperature. Preincubated antibody solutions were applied to the plate, incubated for 1 h and washed. Further, the plates were incubated with goat antirabbit alkaline phosphatase labeled antibodies for 1 h and ELISA was visualized with *p*-nitrophenyl phosphate. The absorbance at 405 nm was measured 2 h after substrate addition.

Percentage of inhibition (% of inhibition) was expressed as: ((A_{405} non-inhibited- A_{405} inhibited)/ A_{405} non-inhibited)) $\times$ 100%.

Primary polyclonal IgG antibodies against major peanut allergens, raised in rabbits, were kindly provided by Dr Maarten Pennings, University Medical Center Utrecht, the Netherlands.

2.6 Anti IgE inhibition ELISA

Concentrations of proteins in peanut extract and cross-linked peanut extract were determined and adjusted to be 1.5 mg/mL by BCA. For ELISA inhibition a pool serum of peanut allergic patients was used. This was prepared by mixing equal amounts of 14 persons' sera with total IgE levels to peanut determined to be: 24.2 kU/L, 86.5 kU/L, 18.6 kU/L, 14.0 kU/L, 5.5 kU/L, >100 kU/L, 4.9 kU/L, 42.2 kU/L, >100 kU/L, 2.3 kU/L, <0.35 kU/L, 1.0 kU/L, 10.01 kU/L, 22.0 kU/L. Peanut extract and cross-linked peanut extract was prepared in tenfold serial dilutions and preincubated for 1 h with the human serum pool at final dilution of 60 times.

Inhibition ELISA was performed as previously described [30]. Briefly, ELISA plates were coated overnight with peanut proteins in PBS. After washing and blocking with 1% BSA in PBS with 0.1% Tween 20, sera incubated with PE, TA, or TT were applied onto plate and incubated for 2 h. Subsequently, plates were washed and incubated with goat antihuman IgE-HRP conjugated antibodies (Pharmingen, San Diego, CA, USA) for 1 h. ELISA was visualized with tetramethylbenzidine substrate solution and stopped after 15 min with 1 M H_2SO_4. and OD at 450 nm was measured. Percentage of inhibition (% of inhibition) was expressed as: ((A_{405} noninhibited A_{405} inhibited)/A_{405} noninhibited) $\times$ 100%.

2.7 Basophil activation test

Basophil activation tests with PE and cross-linked samples were performed as described previously [15]. Heparinized blood samples were taken from three peanut-allergic patients. Patients were selected on the basis of positive skin prick testing to peanuts, documented clinical history of allergy to peanut and positive in vitro testing. IgE levels were assessed with an ImmunoCAP®100 system using ImmunoCAP® code f13 (Pharmacia Diagnostics, Uppsala, Sweden). Levels were considered positive if above 0.35 kAU/L. IgE levels to peanut in three tested patients were determined to be: 56.6 kU/L, 4.72.5 kU/L, and 1.1 kU/L.

2.8 Pepsin digestion

Pepsin digestion of proteins was performed in 0.1 M HCl at containing 2 g/L sodium chloride pH 1.2 with final concentration of proteins to be digested of 0.25 mg/mL (determined by BCA assay) and pepsin activity 2300 U/mL (1 mg/mL) [31]. Aliquots were taken at 1, 5, 10, 15, 30, 45, 60, 120, and 180 min and reactions were stopped with 2 M sodium carbonate. pH after the addition of 2 M sodium bicarbonate was 8.1. Digestion profiles were analyzed on 12% SDS-PAGE under reducing conditions.

2.9 Labeling of cross-linked material and peanut extract with FITC

Cross-linked and native peanut extracts were labeled with FITC according to manufacturer's instructions (Sigma-Aldrich (St. Louis, MO, USA)). Nonreacted FITC was separated from FITC-labeled proteins by gel filtration on PD-10 Desalting Columns (GE Healthcare, Uppsala, Sweden). Protein concentrations in FITC-labeled samples were determined by the Pierce ® 660 nm Protein Assay (Thermo Scientific, Bonn, Germany).

2.10 Growing of Caco-2 cells and transport studies

The TC-7 subclone (ATCC no. HTB-37) of the Caco-2 parental cell line was a generous gift from Monique Rousset (Nancy University, Lorraine, France). Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM + GlutaMAX; Gibco), supplemented with 1% nonessential amino acids, 20% heat-inactivated fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA, USA), 1% of a mixture of penicillin and streptomycin (10 000 units and 10 mg/mL, respectively) and subcultured weekly after reaching approximately 80% confluence. Cultures were maintained at 37°C in an atmosphere of 5% CO_2, 95% air, and at 95% relative humidity. The Caco-2 cells were seeded at 1.6×10^4 cells/insert on polycarbonate membranes (pore size 1 μm), in 24-well plates (Transwell, Corning

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Costar, Cambridge, MA, USA). Cells were cultured for 16 days from passage 18. Only cell monolayers with a transepithelial electrical resistance above 500 Ω were used.

Transport studies were done with 100 μg/mL of FITC-labeled PE or cross-linked PE in DMEM culture medium in 200 μL total volume. Fluorescence in the aliquots was measured using a spectrofluorimeter (FluoroMax®-4, HORIBA Jobin Yvon Inc, NY, USA), and the concentration of labelled protein material was calculated from standard curves of corresponding material.

2.11 Determination of transported Ara h 2 and Ara h 6 across Caco-2 cell monolayer

The concentration of Ara h 2 and Ara h 6 and fragments derived from these proteins in Caco-2 cell basolateral compartments were determined by competitive inhibition ELISA as described before [29]. Briefly, plates were coated with purified Ara h 2 or Ara h 6. Aliquots of peptide containing medium obtained from 2 to 4 h of transcytosis were incubated with appropriate rabbit polyclonal antibodies to a final 10 times dilution of samples. A mixture of antibodies and supernatant samples were added to the well and incubated for 2 h at room temperature. After washing, antirabbit antibody coupled to alkaline phosphatase (ABD Serotec, Oxford, UK) in 1% BSA in PBS-Tween was added to each well and incubated overnight at 4°C. ELISA was developed with *p*-nitrophenyl-phosphate and absorbance at 405 nm was measured. Standard inhibition curves were prepared with antibodies incubated with purified Ara h 2 and Ara h 6 prepared in fivefold dilution series starting from 25 and 10 μg/mL, respectively. Measurements for each sample were done in triplicates. Percentages of inhibition for PE and cross-linked proteins were calculated relatively to the control sample that contained only medium in transport studies.

2.12 In vivo studies

2.12.1 Mice

Female, specific pathogen-free C3H/HeOuJ mice (4 weeks of age), were purchased from Charles River (Lyon, France). Mice were maintained under barrier conditions in filter-topped macrolon cages with wood-chip bedding, at a mean temperature of 23° ± 2°C, relative humidity of 50–55%, and a 12 h light/dark cycle. Drinking water and standard laboratory food pellets were provided ad libitum. No peanut protein was present in the diet. The experiments were approved by the Animal Experiments Committee of Utrecht University, the Netherlands.

2.12.2 Oral sensitization to peanut

Mice were exposed to 6 mg PE ($n = 8$) or cross-linked PE ($n = 5$) with 15 μg cholera toxin (List Biological Laboratories, Inc., Campbell, CA, USA) by an intragastric gavage for 3 consecutive days, and this dosage was repeated once every week for 4 weeks. The control group ($n = 5$) received PBS with cholera toxin. All mice received a challenge of 12 mg PE intragastrically on day 28, and were sacrificed 1 day later. To measure the level of specific antibodies, blood was collected on days 22 and 29 by cheek puncture. Blood samples for measuring the levels of mouse mast-cell protease-1 (mMCP-1) were collected 30 min after oral challenge on day 28 (Fig. 4A).

2.12.3 Induction of oral tolerance to peanut

Mice ($n = 8$) received 1 mg PE or cross-linked PEor PBS via intragastric gavage for 3 consecutive days. This was followed by intraperitoneal immunization with 100 mg PE/alum (Imject, Thermo Scientific, Rockford, IL, USA) 14 and 21 days after the last exposure. Blood was taken on day 34 and subsequently animals were sacrificed. Negative control group received PBS and was injected with PBS/alum.

2.12.4 Measurement of IgE, IgG1, and IgG2a PE-specific antibodies and mMCP-1

PE-specific IgE, IgG1, and IgG2a antibody levels in serum were detected as previously described [32–34] and are expressed as arbitrary units. mMCP-1 was determined using an ELISA kit (Moredun Scientific Ltd., Midlothian, Scotland) and performed according to instructions of the manufacturer.

2.12.5 Cell culture and cytokine measurements

Spleen single-cell suspensions (3.75×10^5 cells in 200 μL of complete RPMI 1640) were incubated with PE (100 μg/mL) or medium alone in 96-well plates for 96 h at 37°C, 5% CO_2 [32, 34]. After centrifugation for 10 min at 150 × *g*, the supernatant was collected and stored at –20°C until analysis. In the culture supernatants, IL-13 and IFN-γ levels were determined by a sandwich ELISA kit (eBioscience, San Diego, USA) according to manufacturer's recommendation.

2.12.6 Statistical analysis

Data in the graphs are presented as mean value for the group ± standard error of the mean. Analyses of data were performed by GraphPad Prism software (La Jolla, CA, USA). Before statistical analysis, all data from in vivo studies were transformed logarithmically and checked for normal distribution. Data were tested by one-way ANOVA with Bonferonni as post hoc test, if not otherwise mentioned. Differences were considered significant when *p*-values were <0.05.

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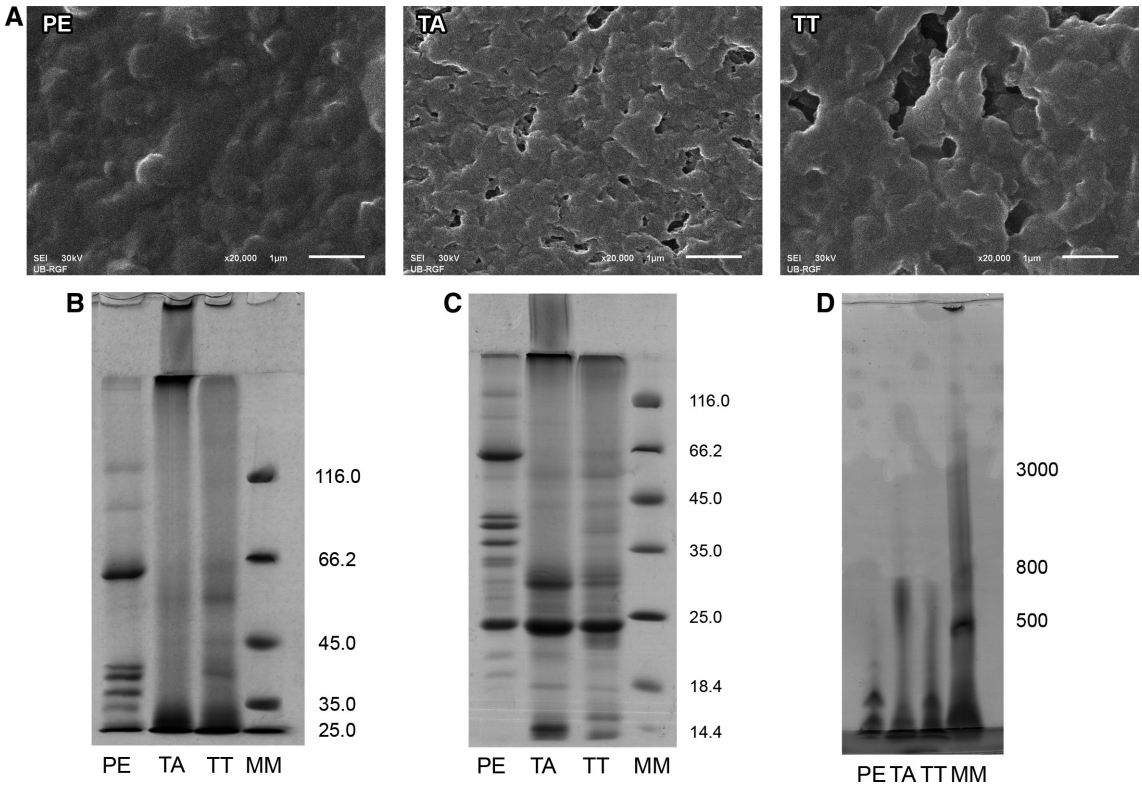

Figure 1. (A) SEM of peanut protein sample, cross-linked with *Agaricus bisporus* (TA) and cross-linked with *Trichoderma reesei* (TT); (B) PE, TA, and TT resolved on 8% SDS-PAGE; (C) PE, TA, and TT resolved on 12% SDS-PAGE; (D) PE, TA, and TT resolved on agarose gel.

3 Results

3.1 Size and aggregation of cross-linked peanut

Electrophoretic and SEM characterization of the cross-linked material showed that treatment with both tyrosinases increased the aggregation of peanut proteins, but treatment with tyrosinase from *A. bisporus* was more effective than tyrosinase from *Trichoderma reseei*. Investigation by SEM of cross-linked PE revealed that this material in comparison to native peanut extract, was in a more aggregated state (Fig. 1A). SDS-PAGE analysis on 8% (Fig. 1A) and 12% gels (Fig. 1B) showed that the obtained material was significantly depleted of bands corresponding to major peanut allergens: Ara h 1 (around 60 kDa), Ara h 2 (doublet of 16 and 18 kDa), Ara h 3 (multiple bands from 10–50 kDa), and Ara h 6 (around 15 kDa). Agarose electrophoresis (Fig. 1C) revealed that both TA and TT samples contained material with higher molecular weight proteins than the native peanut extract. In comparison with peanut proteins, TA was extensively cross-linked, with dominant protein molecular weight ranging over 500 to 3000 kDa, with a majority of proteins being around 800 kDa. TT sample contained proteins of molecular weight up to 800 kDa, with majority of proteins being around 500 kDa.

3.2 Molecular properties and IgE-binding epitopes of cross-linked peanut proteins

Next, in order to provide a profound insight into the immunogenic properties of the material obtained by action of tyrosinases, the ability of material to inhibit anti Ara h 1, Ara h 2, Ara h 3, and Ara h 6 antibodies was investigated (Fig. 2A–D). In all cases, inhibition curves of peanut proteins, TA and TT have the same shape and comparable trends, showing no difference in binding to polyclonal antibodies. Thus, the peanut allergens in the extracts have not been extensively modified in a way that can either disintegrate their 3D structure or alter surface by covalent modifications. In order to prove that treatment with tyrosinases did not alter IgE-binding epitopes of proteins, inhibition of binding of human IgE to peanut proteins was done (Fig. 2E). Results showed that extracts obtained from treatment with these two enzymes maintained IgE-binding epitopes and the same ability to bind to human IgE antibodies. The functionality to elicit basophil degranulation by the extracts was investigated in a basophil activation assay. Upon cross-linking of the IgE antibodies by the allergen, human basophils upregulate surface markers CD203c and CD63. CD63 marker, a measure of anaphylactic degranulation of basophils, was upregulated upon incubation with all three tested samples (Fig. 2F). Thus, the ex vivo ability of

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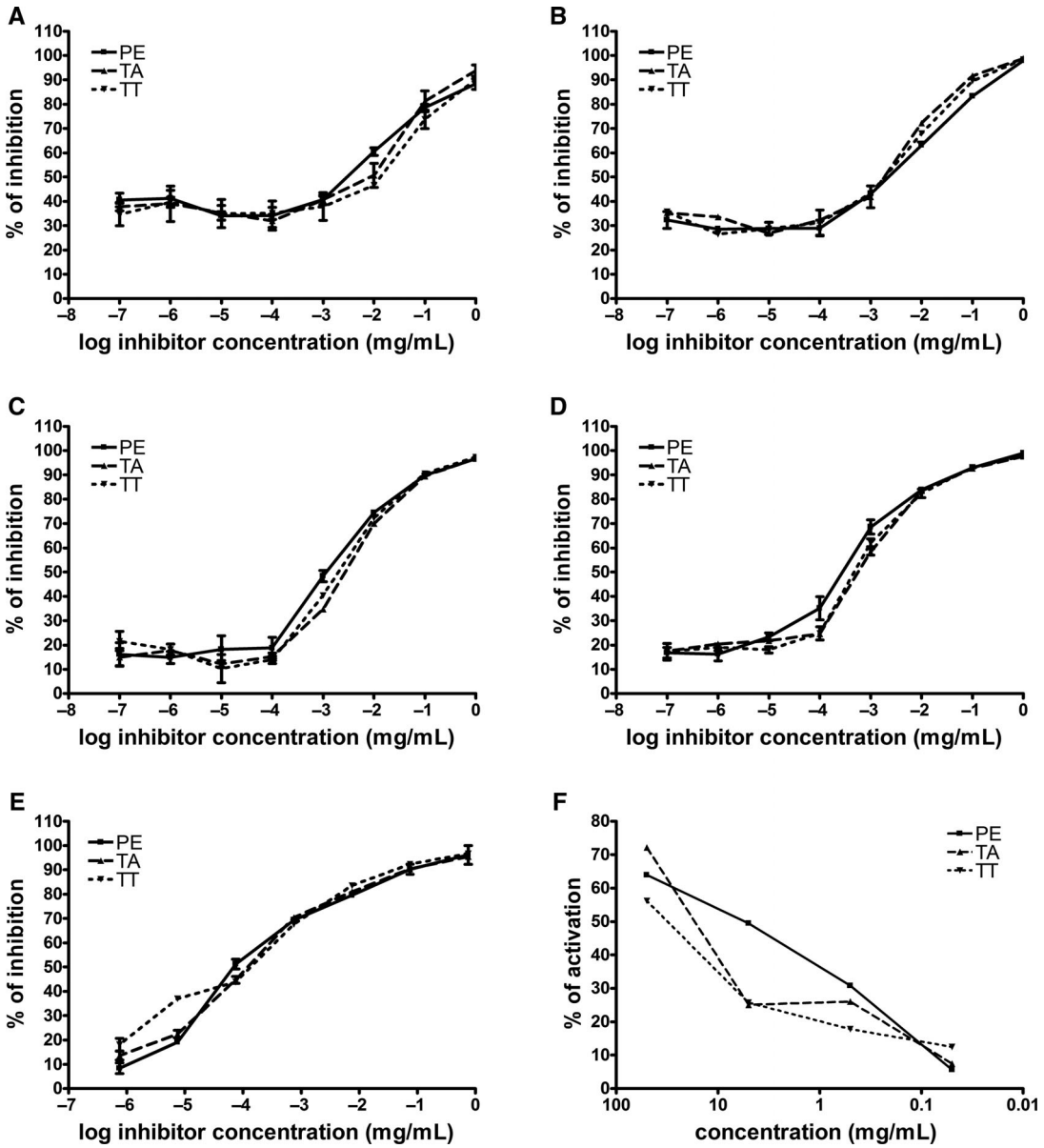

Figure 2. Measurement of PE, TA, and TT binding inhibitory ELISA to: (A) anti-Ara h 1 antibodies; (B) anti-Ara h 2 antibodies; (C) anti-Ara h 3 antibodies, and (D) anti-Ara h 6 antibodies. Preservation of human IgE binding to obtained material: (E) inhibitory ELISA with human serum pool ($n = 14$), (F) basophil degranulation assay. Error bars represent ± SD. Only one representative patient was shown.

tyrosinase cross-linked peanut proteins to activate human basophils was preserved and is comparable to peanut proteins' potency (Fig. 2F).

3.3 In vitro pepsin digestibility of cross-linked peanut proteins

In order to investigate susceptibility to pepsin digestion of the cross-linked peanut proteins, we analyzed digestion of untreated PE and cross-linked peanut proteins by SDS-PAGE. The obtained protein profiles (Fig. 3A–C) revealed a different fate of the proteins among samples: TT material had an electrophoretic profile comparable to unmodified peanut proteins. However, digestion of TA sample revealed that upon pepsinolysis, protein fragments of molecular mass higher than 116 kDa survived over 2-h period.

3.4 In vitro bioavailability of cross-linked proteins

Human intestinal epithelial cells (Caco-2) are used as a model for intestinal uptake and transport of allergenic proteins and therefore we compared the transport efficacy between native and modified food allergens in this model.

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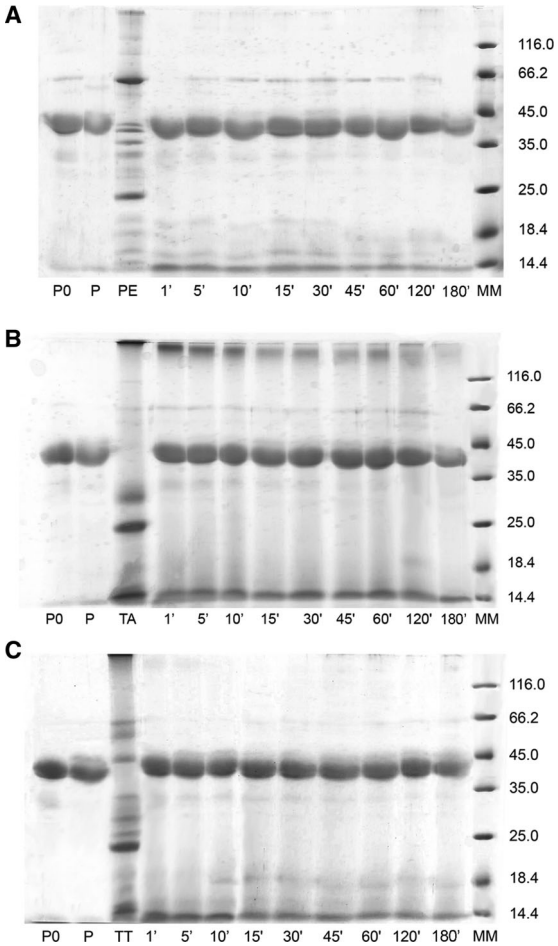

Figure 3. Pepsinolysis of samples: (A) PE, (B) TA, and (C) TT. P0—pepsin at $t = 0$ min, P—pepsin at 180′, PE, TA, TT—samples control. MM—molecular weight markers. Numbers indicate profile at specific timepoints during digestion.

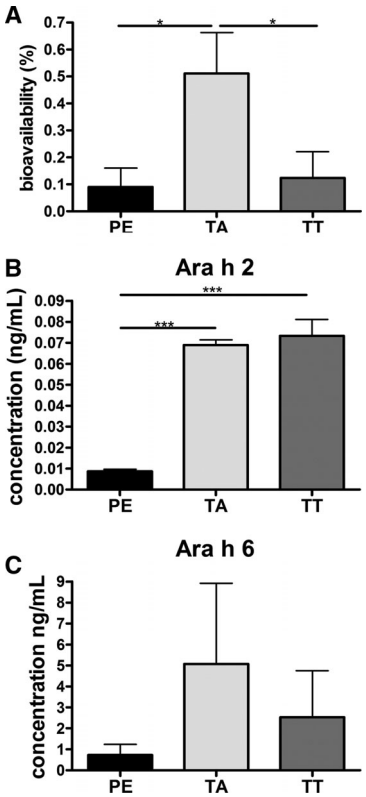

Figure 4. Bioavailability of PE or cross-linked material in Caco-2 cell line: (A) total bioaccesibility; (B) bioavailability of Ara h 2; (C) bioavailability of Ara h 6. $*p < 0.05$, $***p < 0.001$ by *t*-test, based on $n = 3$ replica each. Error bars represent standard error of the mean.

Measurement of bioavailability of cross-linked material showed that TA was transported through the epithelium at a higher rate than TT and PE (Fig. 4A). The transport of peanut proteins was reflected in relatively low bioavailability of the major peanut allergens Ara h 2 and 6. However, the amount of Ara h 2 from TA and TT samples was significantly higher in comparison to PE (Fig. 4B). In contrast, the amount of Ara h 6 (Fig. 4C) in basolateral phase was not significantly different among groups.

3.5 Food allergic responses after intragastric exposure to cross-linked peanut proteins

The sensitizing and allergenic potential of food allergens is commonly tested in different animal models of food allergy [33, 35]. Therefore, an oral animal model of peanut allergy was used to test allergenicity of cross-linked peanut proteins in vivo (Fig. 5A). First, exposure of mice to PE, TT, and TA led to a similar increase in the levels of PE-specific IgE, IgG1, and IgG2a (Fig. 5B) in serum. Second, the levels of IFNγ and IL-13 after restimulation with PE were increased in all exposed groups. However, the level of these cytokines was significantly lower in TT-exposed mice compared to PE-exposed mice. Third, the mucosal mast cell degranulation was assessed by measuring mMCP-1 in serum taken 30 min after intragastric exposure to PE. Sensitization with PE, TT, and TA elicited an equal increase in mMCP-1 (Fig. 5C).

3.6 Oral tolerance induction by cross-linked proteins

The ability of cross-linked material to induce systemic tolerance was investigated in a mouse model of oral tolerance (Fig. 6A). Mice that intragastrically received PE before systemic sensitization with PE showed reduced levels of PE-specific IgE, IgG1, but not IgG2a (Fig. 6B). Treatment with TA-PE led to a decrease only in IgE production and TT-PE lowered both IgE and IgG1 production. Treatment with all materials did not affect production of IgG2a.

At the T cell level, levels of IFN-γ and IL-13 were measured in spleen cell cultures (Fig. 6C). Treatment with PE did not affect production of these cytokines, as well as treatment

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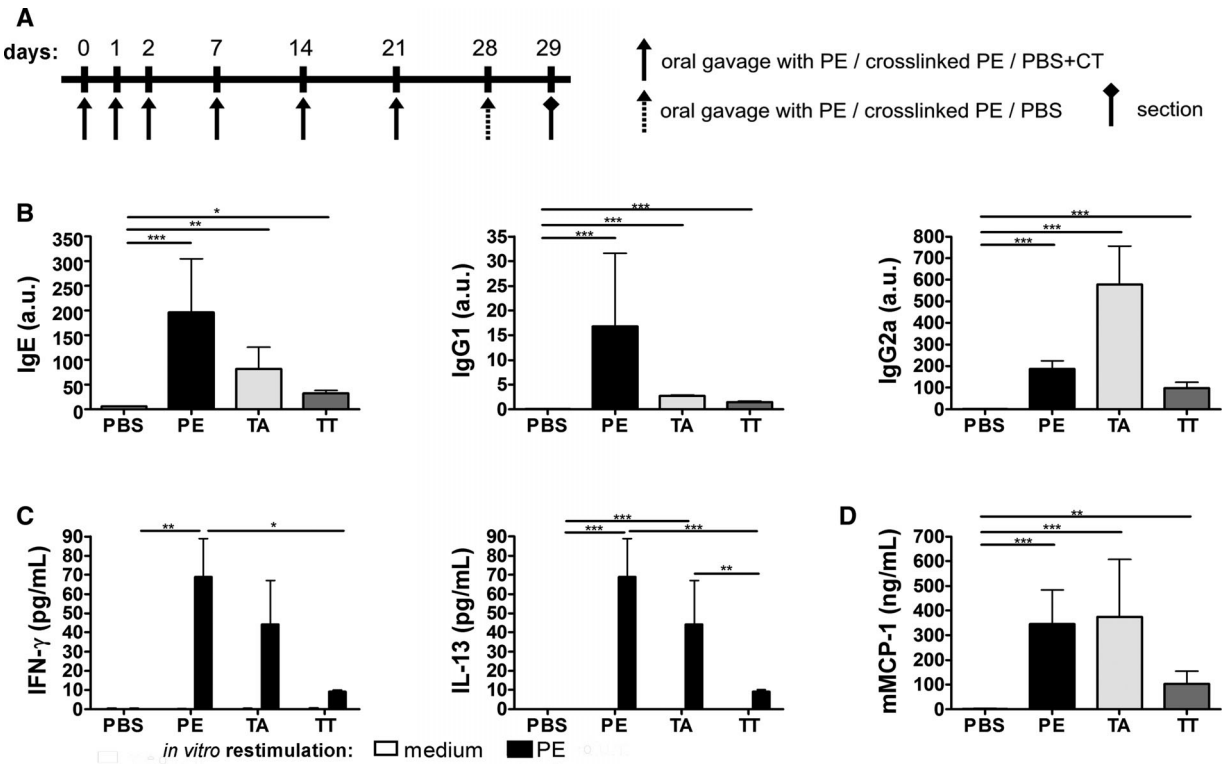

Figure 5. Sensitization experiment: (A) sensitization protocol; (B) IgE, IgG1, and IgG2a on day 29th; (C) IFN-γ measured in spleen-cells' culture; IL-13 measured in spleen-cells' culture; (D) mMCP-1 in serum. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$. Error bars represent standard error of the mean.

with TA. Treatment with TT however increased IFN-γ and decreased the production of IL-13.

4 Discussion

The present work shows that the tyrosinases cross-linked peanut proteins possess allergenic and immunologic properties comparable to the starting material (Table 1). Cross-linking affects size and aggregation of peanut proteins, moderately affects digestibility of proteins and bioavailability of major allergens through a Caco-2 cell monolayer, but eventually does not affect induction of food allergy nor oral tolerance development in mice.

Many studies on cross-linked dietary proteins have shown that through the action of different enzymes allergenic proteins may be altered in such a way to decrease binding to IgE [15, 16, 36, 37]. Material obtained by action of tyrosinases on peanut proteins in our study showed no pronounced change in IgE binding, which is in agreement with previous studies showing moderate effects of cross-linking on IgE-binding properties of peanut allergens: treatment of peanut proteins with polyphenol oxidase (tyrosinase)/caffeic acid and transglutaminase treatment of peanut allergens) led to the formation of cross-linked proteins of high molecular weight without influencing their allergenicity [36, 37]. Major peanut allergens possess many linear IgE-binding epitopes [38–40] and even large conformational changes induced by processing are less likely to influence IgE binding to those regions. We have shown that cross-linked PE obtained after the tyrosinase treatments had similar property (compared to native PE) regarding to binding to polyclonal sera raised against major peanut allergens, as shown in the present study. Hence, the treatment of peanut proteins with tyrosinases preserved the molecular properties of major allergens and the ability to bind IgE and trigger basophil degranulation.

Tyrosinase cross-linked PE showed a different extent of aggregation and stability to pepsin digestion. TA sample showed higher extent of aggregation and survival of fragments of higher molecular weight for a prolonged time in in vitro pepsin digestion. On the other hand, TT sample had aggregates of lower molecular weight and was digested in a similar way as untreated peanut proteins. Hence, it may be expected that these two treatments would behave differently in the GI tract and eventually elicit a different immunological response comparing it to untreated peanut protein. However, the differences in pepsin digestibility of cross-linked proteins could have been compensated for due to propensity of peanut allergens to aggregate after enzymatic digestion. It has been shown previously that the most abundant major peanut

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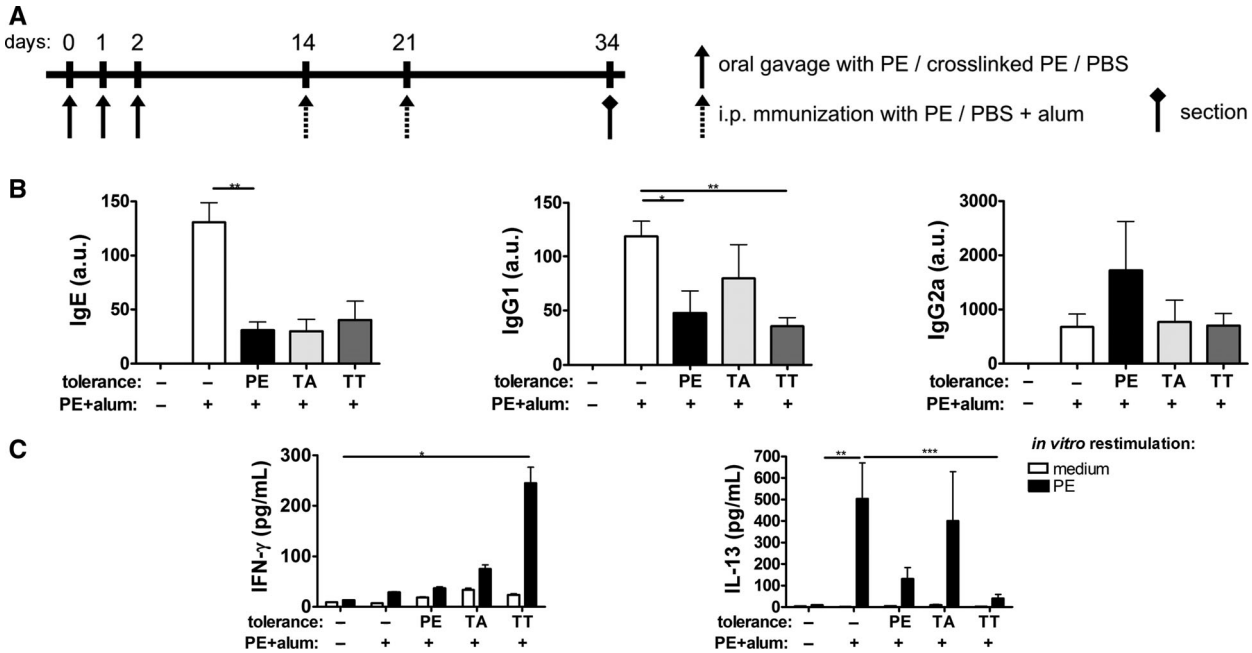

Figure 6. Tolerance induction: (A) oral tolelarnce induction protocol; (B) IgE, IgG1, and IgG2a concentration; (C) IFN-γ measured in spleen-cells' culture; IL-13 measured in spleen-cells' culture. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$. Error bars represent standard error of the mean.

allergen Ara h 1 is an easily digestible protein under physiological conditions [41]. However, pepsin digestion products of Ara h 1 retained sensitizing potential in a Brown Norway rat model [10], a feature lost after separation of pepsin-generated peptides into individual fractions. Thus, the preserved sensitizing capacity of digested Ara h 1 seems to be a consequence of the Ara h 1 peptic peptides being in an aggregated state, resembling the intact molecule.

There are several uptake mechanisms by which intact soluble allergens and their peptides can pass the epithelial border and reach the underlying immune system and induce allergic responses in susceptible individuals. The transcellular transport pathway allows large antigenic molecules to reach the subepithelial compartment and interact with local immune cells [42, 43]. In the present study, we have demonstrated that native PE showed a very low bioavailability in a cellular model of epithelial transcytosis. This implies that peanut allergens, similarly to other dietary proteins prone to aggregation, may also employ routes other than transcytosis through enterocytes (i.e. via M cells associated with Peyer's patches) in order to reach the immune system [4, 42, 43]. Cross-linking and aggregation of major peanut allergens by enzymes did not further change this transepithelial transport. In contrast, peanut proteins demonstrated an increased

Table 1. Overview of data

	PE	TA	TT
Size	10–100 kDa	500–800 kDa	100–500 kDa
Ara h 1, Ara h 2, Ara h 3, and Ara h 6 presence	+	+	+
IgE binding	+	+	+
Basophil degranulation	+	+	+
Pepsinolysis	Partial	Resistant high-molecular-weight aggregates	Similar to PE
Bioaccessibility of peanut proteins	+	↑	+
Bioaccessibility of Ara h 2	+	↑	↑
Bioaccessibility of Ara h 6	+	+	+
Allergenic potential	Mixed Th1/Th2	Mixed Th1/Th2, comparable to PE	Mixed Th1/Th2, IFN-gamma ↓, IL-13 ↓
Tolerance induction (compared with no treatment)	IgE ↓, IgG1 ↓	IgE ↓	IgE ↓, IgG1 ↓, IL-13 ↓, IFN-gamma ↑

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bioavailability upon cross-linking, especially after the *A. bisporus* treatment. We also quantified Ara h 2 and Ara h 6 by competitive inhibition ELISA in Caco-2 cell effluents. Interestingly, the specific action of both tyrosinases facilitated Ara h 2 transport through the Caco-2 monolayer compared to the native PE, yet not influencing transport of Ara h 6. Among major peanut allergens, Ara h 2 and Ara h 6 were the most potent peanut allergens in vivo and the major elicitors of anaphylaxis that accounted for the majority of effector activity in crude peanut extract when assayed with RBL SX-38 cells sensitized with IgE from human peanut allergic sera [44]. Previous reports on transport studies of structurally similar allergens, 2S albumins of Brazil nuts and sesame (Ber e 1 and Ses i 1) revealed that these proteins can be transported intact across Caco-2 cell monolayers [45] Thus, capacity of cross-linked peanut allergens to still induce allergic sensitization may be related to retained (and even facilitated) transport of major peanut allergens. Similarly, it has been shown that heating of purified Ara h 2 led to aggregation of the protein and increased its adsorption to enterocytes [46].

A recent study has shown that a peanut extract rich in proteins may reduce bioavailability of other food allergens. The authors showed that peanut proteins inhibited uptake of Bos d 5, Mal d 1, and Cor a 8 in a Caco-2 cellular model [47]. Certain aromatic ethyl esters and peptides may inhibit Caco-2 cellular uptake of unrelated food allergens as well [48]. This suggests that the epithelial route of entrance for major peanut allergens could be inhibited by the peanut matrix and bulk of nonallergenic peanut proteins, peptides and small molecular weight compounds, i.e. polyphenols, present in the extract [47] which seem to be affected by tyrosinase treatment.

According to our results, aggregation of peanut proteins by cross-linking does not change immunological properties of peanut extract and does not change the induction of (food) allergic responses in animals. It has been shown that heating of purified Ara h 2 led to aggregation of the protein and resulted in an increased allergenicity in vivo [46]. In contrast to these findings, our results showed that peanut proteins treated with tyrosinase did not increase ability to sensitize mice in comparison to untreated peanut proteins. Thus, stable covalent cross-linking and aggregation of proteins prone to aggregate in physiological fluids, may not further promote allergic sensitization. Peyer's patches are macroscopic lymphoid tissue of the GI tract, specialized for uptake of aggregated proteins and particulate matter (i.e. caseins, nonpathogenic bacteria), while soluble proteins (i.e. β-lactoglobulin and α-lactalbumin) pass the transepithelial border by transcytosis through enterocytes [4]. It has been shown that upon aggregation of soluble proteins by pasteurization, the route of entrance of globular proteins changed to Peyer's patches and resulted in an enhancement of allergic sensitization in vivo [4]. In contrast, proteins intrinsically prone to aggregation, i.e. caseins [4, 49] and, according to our study, peanut proteins, did not change immunological properties in vivo upon treatments that promote aggregation of proteins.

A failure to induce oral tolerance in mice may reflect the possible sensitizing capacity of dietary antigens [11]. The present study shows that for both TA and TT cross-linked peanut proteins, the capacity to induce oral tolerance was not compromised. This further supports to our observation that the immunological response to cross-linked peanut allergens is similar to untreated peanuts. Cross-linked PE was transported over the epithelial monolayer as efficient or even more than the native peanut proteins, a feature essential for induction of oral tolerance [50]. Thus, in agreement with the in vitro data demonstrating preserved transport of aggregated peanut proteins across the epithelial barrier, oral tolerance was induced in all treated groups of animals.

In conclusion, in this study, we have shown that enzymatic treatment of peanut proteins by two different tyrosinases yielded high molecular weight and aggregated covalently cross-linked proteins. However, this preserved molecular and immunological features of peanut allergens. The aggregation of peanut allergens by tyrosinases does not reduce specific IgE antibody binding and transepithelial transport of allergens in vitro, does not promote allergic response in vivo, nor compromises the capacity of peanut proteins to induce low dose oral tolerance in mice. Results of our study revealed that covalent cross-linking of proteins otherwise prone to aggregate may be a safe approach, as both sensitizing capacity and potency to induce oral tolerance were not modified by processing. In the future, it would be important to examine effects of peanut allergen cross-linking in a real food system and in a complex food where enzymatic processing can aggregate allergens from different sources.

The authors acknowledge support of the GA No. 172024 of the Ministry of Education and Science of the Republic of Serbia and FP7 RegPot project FCUB ERA GA No. 256716. The EC does not share responsibility for the content of the article. Luka Mihajlovic's research visit to VTT and Maja Krstic's research visit to the Gabriel Lippmann Center were supported by COST Action 928 and COST Action FA1005, respectively. Jelena Radosavljević was an EAACI Clinical Fellowship Award holder.

The authors have declared no conflict of interest.

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Sensitizing potential of enzymatically crosslinked peanut proteins in a mouse model of peanut allergy

Jelena Radosavljevic[1], Emilia Nordlund[2], Luka Mihajlovic[1], Maja Krstic[1], Torsten Bohn[3], Johanna Buchert[2], Tanja Cirkovic Velickovic[1#] and Joost Smit[4]

Institutions:
[1] University of Belgrade, Faculty of Chemistry, Belgrade, Serbia
[2] VTT Technical Research Centre of Finland, Espoo, Finland
[3] Centre de Recherche Public - Gabriel Lippmann, Belvaux, Luxembourg
[4] Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands

Supplementary online material

Treatment of peanut extract with enzymes – Optimizing cross-linking reaction

Peanut protein lyophilisate was dissolved in 100 mM phosphate buffer pH=7.0 and adjusted to a protein concentration of 5 mg/mL. Enzymes were dosed at 100 and 1000 nkat/ g peanut protein according to the measured enzyme activities described in the Experimental section of the manuscript. The reaction times used were 2, 6 and 24 hours and effect of caffeic acid on crosslinking was investigated as well. The enzyme treatments of peanut protein extracts with tyrosinases resulted in an increase in the molecular weight of proteins as analyzed on Criterion Tris-HCl precast 4-20% gradient gels (Biorad, Hercules, CA, USA) (Figure S1A-F).

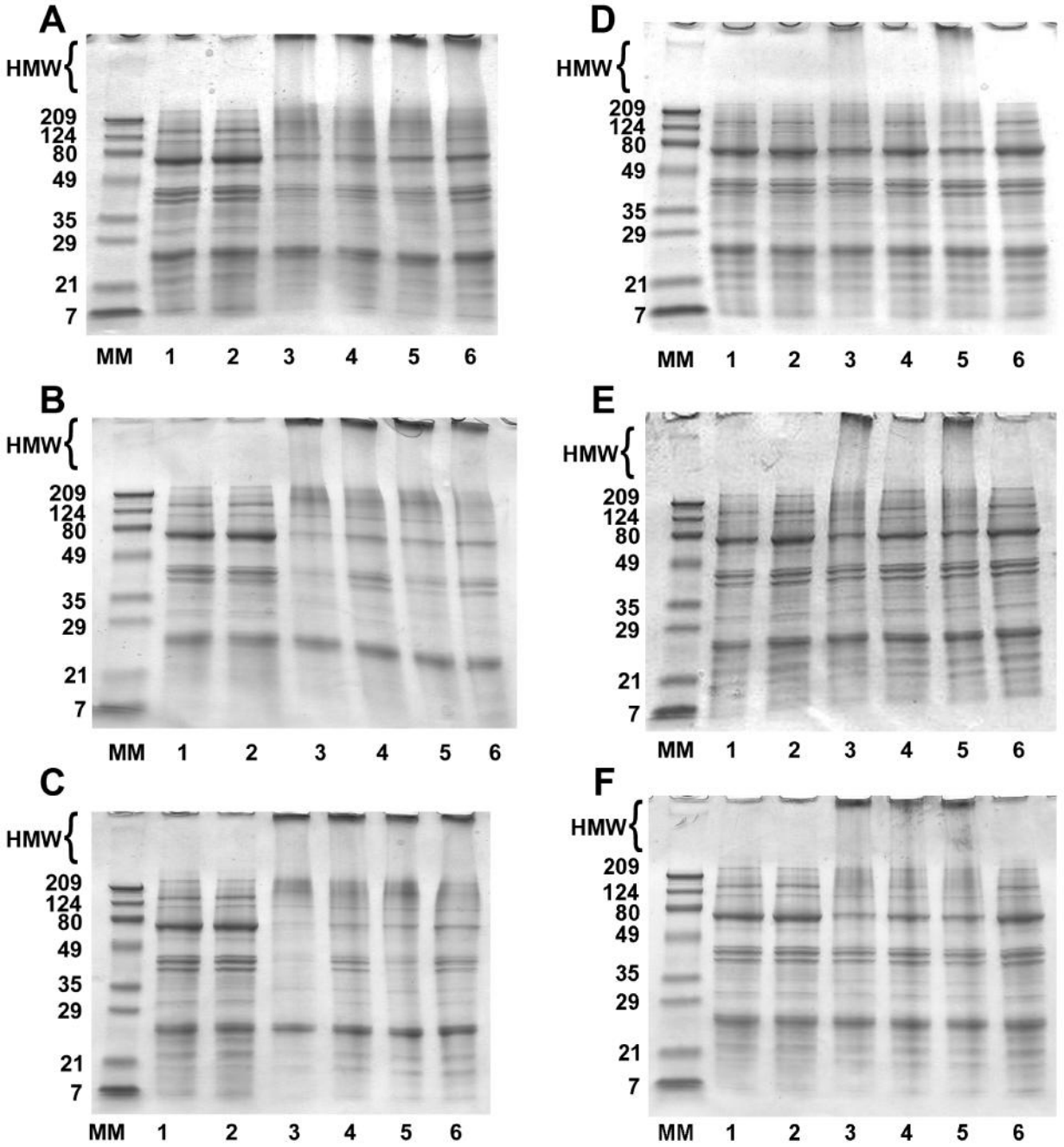

Figure S1: Optimization of conditions for crosslinking peanut proteins with tyrosinases: A, B and C- treatment with *Agaricus bisporus* tyrosinase for 2, 6 and 24 hours; D, E and F- treatment with *Trichoderma resei* tyrosinase for 2, 6 and 24 hours. All gels have the same layout: lane 1 - peanut proteins + 1mM caffeic acid, lane 2 - peanut proteins, lane 3 – 1000 nkat/g of enzyme + 1mM caffeic acid + peanut proteins, 4 – 1000 nkat/g of enzyme + peanut proteins, 5 – 100 nkat/g of enzyme + 1mM caffeic acid + peanut proteins, 6 – 100 nkat/g of enzyme + peanut proteins, MM – molecular weight markers, HMW – high molecular weight proteins.

Preliminary reactions showed that the best results (regarding the extent of the crosslinking and the size of crosslinks) were obtained with enzyme activities of 1000 nkat/g protein and a 24 h reaction time, for both of the examined enzymes. The effect of pH on modulation of crosslinking was investigated further: for *Agaricus bisporus* tyrosinase reactions were performed at pH=6 and pH=7, and for *Trichoderma resei* tyrosinase reactions and were performed at pH=7 and pH=8, in the presence, or absence of caffeic acid as a mediator for 24 hours (Figure S2):

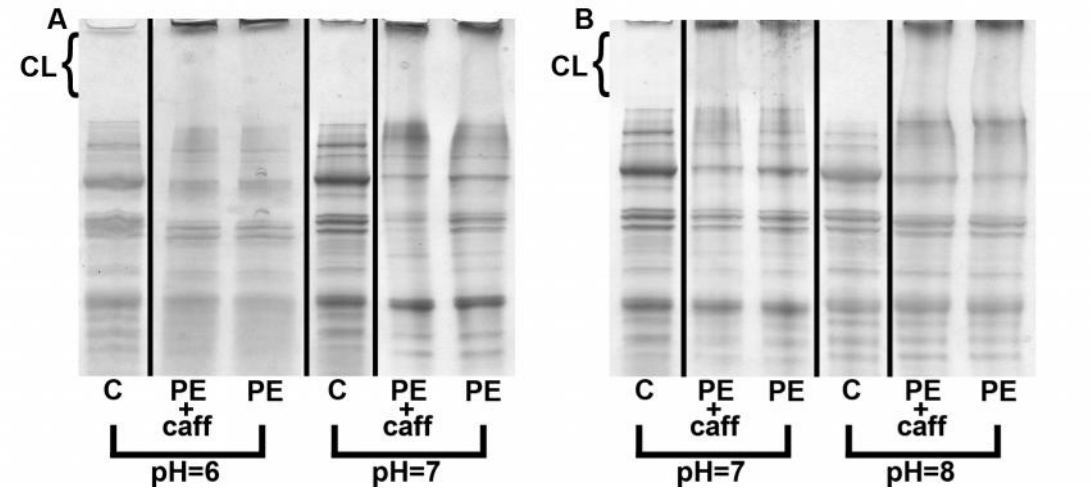

Figure S2: Modulation of crosslinking by pH value. A - treatment with *Agaricus bisporus* tyrosinase, B – treatment with *Trichoderma reesei* tyrosinase. C – peanut proteins (control), PE+caff - peanut proteins treated in the presence of caffeic acid as mediator, PE -peanut proteins treated with no mediator. CL – higher molecular weight crosslinks.

Based on results of optimizations, the following results were chosen for large scale production of crosslinked material: enzyme activity was set to be 1000 nkat/g protein and incubation took 24 hours. Preliminary results suggested that addition of caffeic acid did not enhance crosslinking reaction. After determination of the phenolics content in peanut extract (~6 mM of gallic acid equivalents) it was chosen to omit caffeic acid as a mediator in crosslinking reaction. Reactions were performed at pH=7 (crosslinking with *Agaricus bisporus* tyrosinase) and pH=8 (*Trichoderma reesei* tyrosinase).