PAPER

Peanut protein structure, polyphenol content and immune response to peanut proteins *in vivo* are modulated by laccase†

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Food texture can be improved by enzyme-mediated covalent cross-linking of different food components, such as proteins and carbohydrates. Cross-linking changes the biological and immunological properties of proteins and may change the sensitizing potential of food allergens. In this study we applied a microbial polyphenol oxidase, laccase, to cross-link peanut proteins. The size and morphology of the obtained cross-linked proteins were analyzed by electrophoresis and electron microscopy. Structural changes in proteins were analyzed by CD spectroscopy and by using specific antibodies to major peanut allergens. The bioavailability of peanut proteins was analyzed using a Caco-2 epithelial cell model. The *in vivo* sensitizing potential of laccase-treated peanut proteins was analyzed using a mouse model of food allergy. Finally, peanut polyphenols were analyzed by UHPLC-MS/MS, before and after the enzymatic reaction with laccase. Laccase treatment of peanut proteins yielded a covalently cross-linked material, with the modified tertiary structure of peanut proteins, improved bioavailability of Ara h 2 (by 70 fold, $p < 0.05$) and modulated allergic immune response *in vivo*. The modulation of the immune response was related to the increased production of IgG2a antibodies 11 fold ($p < 0.05$) and reduced IL-13 secretion in *in vitro* cultured splenocytes 7 fold ($p < 0.05$). Analysis of the peanut polyphenol content and profile by HPLC-MS/MS revealed that laccase treatment depleted the peanut extract of polyphenol compounds leaving mostly isorhamnetin derivatives and procyanidin dimer B-type in detectable amounts. Treatment of complex food extracts rich in polyphenols with laccase results in both protein cross-linking and modification of polyphenol compounds. These extensively cross-linked proteins have unchanged potency to induce allergic sensitization *in vivo*, but certain immunomodulatory changes were observed.

1. Introduction

Protein cross-linking in food may occur naturally, during processing, or by exposure to environmental pollutants.[1–5] Enzymatic cross-linking of proteins that leads to formation of high molecular weight aggregates of proteins (and/or other compounds present in food, such as polysaccharides) is often exploited in the cereal, dairy, meat, and fish processing industries in order to improve the mechanical and functional properties of food.[2] Although enzymatic cross-linking of proteins has been carried out extensively, little is known about the health risks of these process-modified food proteins of a very high molecular weight.

Tailoring of the protein structure is necessary for creation of new functional food properties, but brings a risk of creating foods with changed immunogenic potential. Therefore, new proteins and protein derivatives need to be tested before they are released into the food market in order to provide assessment of the allergenic potential of novel foods regarding prevention of development of *de novo* hypersensitivity and prevention of an increase of the allergenic potential of allergens already present in foods.[6]

The IgE binding capacity of processed and modified foods or proteins is the most common method for examination of how food processing affects the allergenicity of food allergens. How processing affects the sensitization capacity is generally studied using animal models of food allergy by administration of purified food proteins, food extracts or allergens present in their natural food matrix.[7–9]

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Peanut is widely used in the human diet, especially in vegetarian diets, and is a major legume for the food industry. Peanut proteins are abundantly available and can be treated by enzymatic or chemical cross-linking in order to develop films, fibers, nanoparticles and other types of materials for various applications.[4]

Peanut proteins also present a serious health risk, as peanut allergy is the most prevalent food allergy in adults, with an estimated 1–2% of the total population and up to 8% of the children population showing allergic symptoms to peanuts.[10] It is also a frequent cause of fatal anaphylactic reactions to foods.[11]

There are several reports on the effect of enzymatic cross-linking on human IgE binding to cross-linked peanut allergens. Transglutaminase was found to reduce the immunoglobulin binding of peanut allergens in peanut flour dispersions containing casein.[12] Treatment of peanut proteins with transglutaminase was shown to result in changed protein properties with the creation of high molecular mass oligomers and polymers, but without significant changes in immunogenic responses.[13] Similar results have been obtained by treatment of peanut allergens with mushroom tyrosinase/caffeic acid and tyrosinase from *T. reesei*.[9,14]

Several recent studies have examined the effects of enzymatic cross-linking on the sensitizing potential of food proteins and the results have been conflicting. In two recently published studies, treatment of peanut proteins with tyrosinase, or milk casein with transglutaminase, did not change the protein sensitizing capacity in animal models of food allergy.[9,15] However, laccase treatment of a whey protein, beta-lactoglobulin, promoted both the immunogenicity and allergenicity of the major cow's milk allergen in mice.[8]

Laccases (benzenediol : oxygenoxidoreductases) are a group of copper-containing enzymes currently in focus due to their numerous applications in bioremediation and textile dye formation,[16] as well as in food technology.[17] Among other applications, laccases are showing promise in the baking industry, fruit processing and the wine industry. Most industrial laccases are of fungal and microbial origin, are cheap to produce on a large scale, and are of sufficient robustness for immobilization and similar procedures.[18]

The goal of this work was to elucidate the effects of laccase-catalysed cross-linking of peanut allergens on their structure, bioaccessibility and capacity to induce food allergy *in vivo*. Due to the fact that laccase is an oxidoreductase which may use intrinsic polyphenols as auxiliary substrates in the cross-linking reaction, we have also analyzed the composition of peanut polyphenols before and after the enzymatic reaction.

2. Materials and methods

2.1 Materials

Unless otherwise stated, all chemicals and antibodies were obtained from Sigma (St Louis, MO, USA). Acetonitrile and acetic acid (both MS grade) were purchased from Merck (Darmstadt, Germany). Ultra-pure water (TKA MicroPure water purification system, 0.055 μS cm^{-1}, Thermo Fisher, Bremen, Germany) was used to prepare the standard solutions and blanks. Syringe filters (13 mm, PTFE membrane 0.45 μm) were purchased from Supelco (Bellefonte, PA, USA). Phenolic standards were purchased from Fluka (Buch, Switzerland). Laccase from *Trametes hirsuta* was purified and characterized as described earlier.[19] The enzyme was purified to electrophoretic homogeneity. Activity of the purified enzyme was 12 195 nkat mL^{-1}. Laccase activity was measured according to Niku-Paavola *et al.*,[20] using ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) as the substrate. Protein concentrations were determined by the bicinchoninic acid assay[21] unless stated differently. Raw in-shell peanuts (*Arachis hypogea* L.) of the runner variety were bought from a major local supplier and were used for all extract preparations. One batch of peanuts was used for the preparation of all extracts.

2.2 Preparation of peanut extract and determination of the phenolic content of the peanut extract

Peanut extract was prepared as previously described.[9] The concentration of total phenolics in the peanut protein extract was determined spectrophotometrically according to Ainsworth *et al.*,[22] with gallic acid as the standard. The enzymatic reaction was stopped by freezing the sample at −20 °C for less than 10 min. The extract was clarified by filtration through a Buchner funnel equipped with Whatman No. 1 filter paper and the clear preparation was lyophilized and used for further investigation.

2.3 Treatment of the peanut extract with laccase

Peanut protein lyophilisate was dissolved in 100 mM sodium phosphate buffer at pH 7.00 and adjusted to a protein concentration of 5.00 mg mL^{-1} by the Bio-Rad DC protein assay kit (Bio-Rad, Richmond, CA, USA). The enzyme was dosed at 1000 nkat per gram of peanut protein according to the measured enzyme activity described above. The reaction was performed on a thermoshaker set to 300 rpm for 24 h at 37 °C. The reaction was stopped by freezing the sample at −20 °C and the extract was subsequently lyophilized.

2.4 Analysis of cross-linked proteins

2.4.1. **Agarose electrophoresis.** In order to determine the molecular masses of the cross-linked products, a modified electrophoresis protocol was used, performed on 1% agarose gel, with custom molecular markers made using mouse myofibrilar proteins, as previously described.[23] A Ruby SE600 system (Hoefer, Molliston, MA, USA) was used for agarose electrophoresis. Gels were stained with Coomassie Brilliant Blue R250 (Serva, Heidelberg, Germany).

2.4.2. **Scanning electron microscopy (SEM).** Control and treated peanut extracts were visualized under a scanning electron microscope JSM-6610 (JEOL, Tokyo, Japan). The solutions of the extracts were adjusted to 1.00 mg per mL protein content, and 40 μL was applied to the probes. After drying and

gold-plating, the images were acquired at 10 000× and 20 000× magnifications and at 30 kV voltage.

2.4.3. CD spectroscopy. To investigate the differences in the secondary and tertiary structures of proteins in peanut extract and cross-linked peanut extracts, their far UV and near UV circular dichroism (CD) spectra were acquired using a JASCO J-815 spectropolarimeter (JASCO, Tokyo, Japan) at 25 °C in 1 nm steps at a rate of 50 nm min^{-1} using 0.01 cm and 1.00 cm path-length quartz cuvettes, respectively. The spectra were collected over the wavelength range 180–260 nm for far UV and 260–320 nm for near UV. Each spectrum was acquired four times, and the results were averaged. The scans were then corrected for buffer. The materials at the protein concentration of 1.20 mg mL^{-1} in 10 mM phosphate buffer (pH 7.00) were used for CD measurements.

2.5 LC/MS of peanut phenolic compounds

Phenolic compounds were extracted from lyophilized untreated and laccase-treated peanut extracts. For this purpose, 20.0 mg of the preparations were extracted twice with 500 μL of 80% aqueous ethanol.[24] Ethanolic extracts from subsequent extractions were combined and submitted for LC/MS analysis.

2.5.1. Determination of phenolic compounds in peanut extract and laccase-treated peanut extract. Separation of compounds was performed using a liquid chromatography system that consisted of a quaternary Accela 600 pump and an Accela autosampler, connected to a linear ion trap–Orbitrap hybrid mass spectrometer (LTQ OrbiTrap XL) with a heated-electrospray ionization probe, HESI-II (Thermo Fisher Scientific, Bremen, Germany).

A Syncronis C18 column (100 × 2.1 mm, 1.7 μm particle size) from Thermo Fisher Scientific (Bremen, Germany) was used as the analytical column used in phenolic quantification. The mobile phase consisted of (A) water + 0.01% acetic acid and (B) acetonitrile. A linear gradient program at a flow rate of 0.25 mL min^{-1} was used: 0.0–1.0 min 2% B, 1.0–14.0 min from 2% to 98% (B), 14.0–14.1 min from 98% to 2% (B), then 2% (B) for 5 min. The injection volume was 5 μL. The mass spectrometer was operated in negative mode. Parameters of the ion source were as follows: source voltage 4.5 kV, capillary voltage −4 V, tube lens voltage −59.11 V, capillary temperature 275 °C, sheath and auxiliary gas flow (N$_2$) 30 and 7 (arbitrary units). The MS spectra were acquired by full-range acquisition covering 120–1000 m/z. A data-dependant scan was performed for the fragmentation study by deploying collision induced dissociation (CID). The normalized collision energy of the CID cell was set at 35 eV.

Xcalibur software (version 2.1) was used for instrument control, data acquisition and data analysis. Phenolic compounds were identified according to their spectral characteristics: mass spectra, accurate mass, characteristic fragmentation and characteristic retention time. The data were verified by comparing with the data obtained by other authors.[25–27]

Quantification of select compounds was carried out according to the exact mass search method (±5 ppm) by comparing the retention times and the exact mass of the available standards. The total amount of each compound was evaluated by integration of the peak area and is expressed as mg kg^{-1}.

2.5.2. Preparation of standard solutions of phenolic acids. A 1000 mg L^{-1} stock solution of a mixture of all phenolic standards was prepared in methanol. Dilution of the stock solution with methanol yielded the working solution of concentrations 0.025, 0.050, 0.100, 0.250, 0.500, 0.750, and 1.000 mg L^{-1}. Calibration curves were obtained by plotting the peak areas of the compounds identified relative to the peak area against the concentration of the standard solution. Calibration curves revealed good linearity, with R^2 values exceeding 0.99 (peak areas $vs.$ concentration).

2.6 Inhibition ELISA with antibodies specific to individual peanut allergens (Ara h 1, 2, 3, 6)

ELISA plates were coated overnight with 100 μL of untreated peanut extract in PBS (concentration: 50 μg mL^{-1}), and blocked with 1.00% BSA in 0.10% Tween 20 in Tris-buffered saline (TTBS) pH = 7.40 for 2 h. Peanut cross-links were prepared at 10 fold serial dilutions (range from 1–10^{-7} mg mL^{-1}) and preincubated for 1 h with antibodies against Ara h 1, Ara h 2, Ara h 3 and Ara h 6 at room temperature. Blocking solution was mixed with TTBS and the preincubated antibody solutions were added to the plate (100 μL per well) and incubated for 1 h. After washing with TTBS, the plates were incubated with goat anti-rabbit alkaline phosphatase-labelled antibodies for 1 h, at a dilution of 1 : 10 000. ELISA was visualized with 100 μL of 1.0 mg mL^{-1} sodium 4-nitrophenyl phosphate (4-NPP) in diethanolamine (DEA) buffer. The absorbance at 405 nm was measured 2 h after substrate addition.

The percentage of inhibition (% of inhibition) was expressed as (A_{405} noninhibited − A_{405} inhibited)/A_{405} noninhibited × 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. All the experiments were performed in triplicate. IC$_{50}$ values were calculated by using OriginPro 8.5.1 (OriginLab Corporation, Northampton, MA, USA).

2.7 Transepithelial transport in a Caco-2 cell model of the epithelium

2.7.1. Labelling of cross-linked material and peanut extract with fluorescein isothiocyanate (FITC). The material for transport studies was labelled with FITC according to the manufacturer's instructions. Removal of non-reacted FITC was done by desalting on PD-10 columns (GE Healthcare, Oxford, UK).

2.7.2. Culturing of Caco-2 cells. The TC-7 subclone (ATCC No. HTB-37) of the Caco-2 parental cell line used in this study was kindly provided by Monique Rousset (Nancy University, Nancy, France). Caco-2 cells were grown as described previously.[9] After passage 18, cells were cultured for 16 days in 24-well plates of 6.5 mm diameter and 0.4 μm pore sizes (Transwell, Corning Costar, Cambridge, MA, USA).

2.7.3. Transport studies. For transport studies only cell monolayers with a transepithelial electrical resistance (TEER) above 500 Ω were used. Two hundred microliters of FITC-labelled material in culture medium (100 μg mL^{-1}) was applied to the apical side of the monolayer. The protein concentration was measured by the Pierce 660 nm protein assay (Thermo Scientific, Bremen, Germany). Fluorescence in the effluents was measured using a spectrofluorimeter (FluoroMax – 4, HORIBA Jobin Yvon Inc, Edison, NJ, USA), and the concentration of the labelled protein material was calculated from the standard curves of the corresponding material. Experiments were performed in triplicate.

2.7.4. Ara h 2 and 6 measurement in effluents. The concentrations of Ara h 2 and Ara h 6 in transcellular medium were determined by ELISA as described before.[9,28] Briefly, high bonding plates were coated with purified Ara h 2 or Ara h 6. Aliquots from the basolateral compartment obtained after 4 h of incubation were incubated with appropriate rabbit polyclonal antibodies in 96-well plates. A mixture of antibodies and supernatant samples was added to the well and incubated for 2 h at room temperature. After washing, the anti-rabbit antibody coupled to alkaline phosphatase (ABD Serotec, Oxford, UK) in phosphate buffered saline supplemented with 1.00% BSA and 0.10% Tween 20 (TPBS) was incubated overnight at 4 °C. ELISA was developed with 4-NPP in DEA buffer. The absorbance was measured at 405 nm. Standard inhibition curves were obtained with antibodies incubated with Ara h 2 and Ara h 6 prepared in 5 fold dilution series (starting concentrations 25 and 10 μg mL^{-1}, respectively). The percentages of inhibition for peanut extract proteins were calculated relative to the sample containing only medium. Experiments were performed in triplicate.

2.8. In vivo experiments

For in vivo studies, female pathogen-free C3H/HeOuJ mice (4 weeks of age) were used (Charles River, Lyon, France). The animals were maintained at a temperature of 21 °C to 25 °C, a relative humidity of 50–55%, and a 12 h light/dark cycle. No peanut proteins were present in the diet, and standard laboratory food pellets and drinking water were provided ad libitum. Mice experiments were performed according to the Animal Ethics Committee of Utrecht University and national Dutch guidelines that were in force at the time of the study (Dutch Animal Experimentation Act and EC Directive 86/609/EEC). The study was approved by the Animal Ethics Committee of the Utrecht University, the Netherlands (Approval number: 2009.III.076.1).

Phosphate buffered saline (PBS), 6 mg of peanut extract in PBS (n = 8) or cross-linked peanut proteins (n = 5) in PBS were co-administered with 15 μg of cholera toxin (List Biological Laboratories, Inc, Campbell, CA, USA) to mice by intragastric gavage on days 0, 1, 2, 7, 14 and 21. On day 28 all the groups were intragastrically challenged with 12 mg of peanut extract in PBS and were sacrificed a day later. Peanut extract in PBS was prepared by dissolving the lyophilized peanut extract (section 2.2) in PBS.

For measuring mMCP-1, blood samples were collected by cheek puncture 30 min upon challenge. mMCP-1 was determined using an ELISA kit (Moredun Scientific Ltd, Midlothian, Scotland) and performed according to the instructions of the manufacturer. The levels of peanut-specific IgE, IgG1, and IgG2a antibodies in sera on day 29 were detected as previously described and are expressed as arbitrary units (a.u.).[29,30]

Production of IFN-γ and IL-13 by splenocytes of sensitized animals upon stimulation with peanut proteins was investigated as previously described.[9,29,31]

2.9. Statistics

Data were compared using Student's *t*-test using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Values in the graphs are represented as mean value ± standard error of the mean, if not mentioned differently. Data from in vivo studies were transformed logarithmically and checked for normal distribution before analysis. Differences were considered significant when *p*-values were <0.05.

3. Results

3.1 Laccase treatment of peanut protein extracts results in protein cross-linking and influences the protein structure

The enzymatic treatment of peanut protein extracts with laccase resulted in an increase of the molecular weight of the peanut proteins. In order to obtain an estimate of the molecular mass of the cross-linked peanut protein aggregates, electrophoresis using agarose gel was performed (Fig. 1A).

The products obtained by the action of laccase had very high molecular weights of around 800 kDa. Protein bands of at least 1000 kDa could also be observed, as well as larger aggregates.

Scanning electron microscopy (SEM) was further employed to obtain information regarding the macroscopic properties of the modified proteins. SEM showed that the laccase cross-linked sample exhibited a sponge-like structure and the formation of dense structures of cross-linked aggregates (Fig. 1B).

The near UV CD spectra showed profound differences in the tertiary structure among the samples (Fig. 1C). The shape and magnitude of the near CD spectrum are usually distorted by differential light scattering and absorption flattening which arise from the high concentrations of protein molecules in protein aggregates. In contrast to the near UV CD spectrum, very slight differences in the far UV CD spectrum were observed indicating no significant alterations of the secondary structures of laccase-treated proteins (Fig. 1D). Hence, CD spectrometry showed that the laccase treatment of peanut proteins did not disturb the secondary structure of proteins, but mostly affected the tertiary structure of peanut proteins.

3.2 Modification of epitopes of major peanut allergens by laccase

Changes of the tertiary structure induced by laccase cross-linking may affect the antigenic epitopes of major peanut
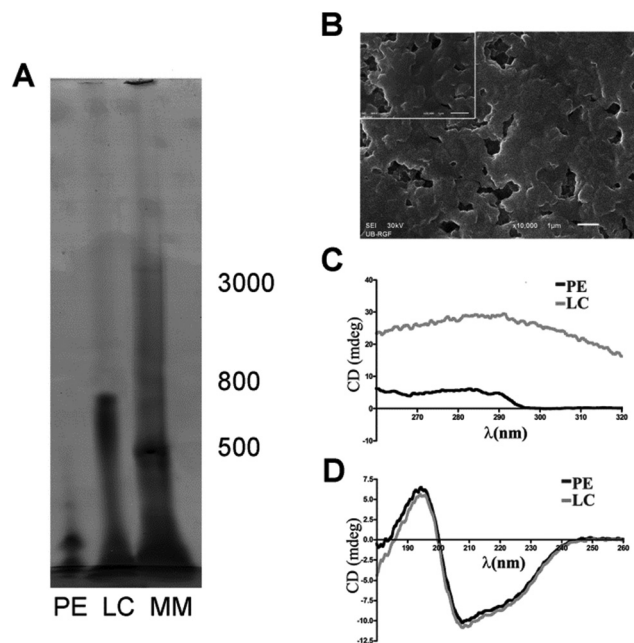
Fig. 1 Characterization of the obtained cross-linked material: A – agarose electrophoresis: MM – molecular weight markers (kDa); cross-linked proteins are clearly visible in the laccase-treated sample; B – scanning electron microscopy image of treated peanut extract under 10 000× and 20 000× magnifications (insert); C and D – circular dichroism spectra of peanut extract and cross-linked material in near (C) and far (D) UV range. PE – untreated peanut extract, LC – laccase-treated peanut extract. All the experiments were performed in triplicate.

allergens. In order to assess epitope modification of individual allergens, inhibitory ELISA with specific peanut allergen antibodies was performed. IC_{50} values were determined as a measure of sample potency to inhibit binding of specific peanut allergen antibodies raised against Ara h 1, Ara h 2, Ara h 3 and Ara h 6 (Fig. 2 and Table 1). The most extensive modification of epitopes could be observed for Ara h 3 and Ara h 6. In the linear range of the inhibitory curves inhibitory potencies were significantly different ($p < 0.01$ for Ara h 3 and $p < 0.05$ for Ara h 6), for the laccase-treated compared to the non-treated proteins (Fig. 2C and D). IC_{50} values obtained for Ara h 1 and Ara h 2 were similar to the native peanut extract sample and the laccase-treated sample and these two allergens seemed to be unaffected by the cross-linking treatment.

3.3 Epithelial transport of cross-linked peanut proteins

Epithelial transport of cross-linked peanut proteins was analyzed in a Caco-2 cell monolayer model system by analyzing the transport of FITC-labelled proteins and determining the specific allergen concentration (Ara h 2 and Ara h 6) by inhibitory ELISA. No statistically significant change in the basolateral compartmental concentration of FITC-labelled peanut proteins in the laccase-treated peanut extract sample was observed (Fig. 3A). In contrast, Ara h 2 showed a 70 fold increased passage through the Caco-2 monolayer after enzyme
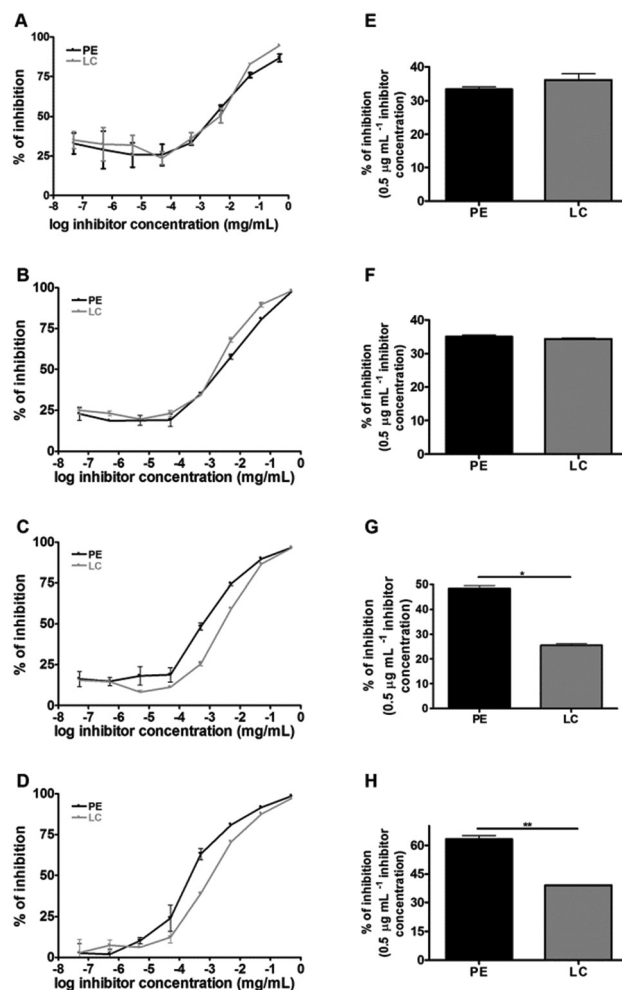
Fig. 2 ELISA inhibition of rabbit anti-Ara h 1 (A), rabbit anti-Ara h 2 (B), rabbit anti-Ara h 3 (C) and rabbit anti-Ara h 6 (D) antibodies by peanut extract (PE) and cross-linked peanut proteins (LC) (data are presented as mean ± SD). The ELISA inhibition values of 0.5 µg mL⁻¹ inhibitor concentration (linear part of inhibition curve) for anti-Ara h 1 (E), anti-Ara h 2 (F), anti-Ara h 3 (G) and anti-Ara h 6 antibodies (H).* $p < 0.05$, ** $p < 0.01$. Experiments were performed in triplicate.

Table 1 IC_{50} values of ELISA inhibition of rabbit-specific antibodies to peanut allergens binding to the peanut extract-coupled microtiter plate (µg mL⁻¹ inhibitor). Values are means of 3 replications ± standard error of the mean. PE – untreated peanut extract, LC – laccase-treated peanut extract

| Inhibitor | IC_{50} values of competitive ELISA with peanut allergen-specific polyclonal antibodies | | | |
	anti-Ara h 1	anti-Ara h 2	anti-Ara h 3	anti-Ara h 6
PE	0.89 ± 0.37	1.25 ± 0.17	0.49 ± 0.15	0.28 ± 0.07
LC	0.46 ± 0.19	0.62 ± 0.07	2.47 ± 0.09	1.14 ± 0.06

treatment (Fig. 3B) ($p < 0.001$), while for allergen Ara h 6, a trend towards an increased transcytosis could be observed, but it was not statistically significant (Fig. 3C).
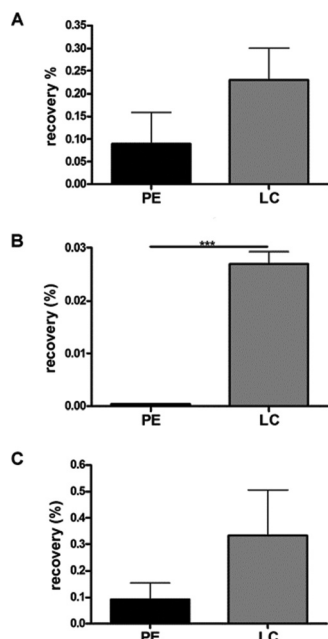
Fig. 3 Epithelial transport of total FITC-labelled peanut protein (A), Ara h 2 (B) and Ara h 6 (C). Results are expressed as % of recovery in the apical phase after 4 h. The experiment was performed in triplicate. ***$p < 0.001$. PE – untreated peanut extract, LC – laccase-treated peanut extract.

3.4. *In vivo* allergenicity of laccase cross-linked peanut allergens

The laccase-cross-linked peanut extract sensitized mice and induced similar production of peanut-specific IgE and IgG1, but a higher production of IgG2a antibodies (Fig. 4) compared to the untreated peanut extract. Upon oral challenge with peanut extract, similar levels of mMCP-1 were measured in mice sensitized with untreated peanut extract or laccase-treated peanut proteins. In contrast, a decreased production of

both Th1 (IFN-γ) and Th2 (IL-13) cytokines in splenocytes of animals sensitized with laccase-treated peanut extract, compared to the untreated peanut extract, was observed in the cultured splenocytes stimulated with the untreated peanut extract.

3.5 Analysis of phenolic compounds of peanut extracts after the action of laccase

The prepared peanut extract was very rich in polyphenolic compounds. The content of polyphenols, expressed as gallic acid equivalents, was 1.00 ± 0.20 mg mL^{-1} in peanut extract that was adjusted to 5.00 mg per mL of peanut proteins.
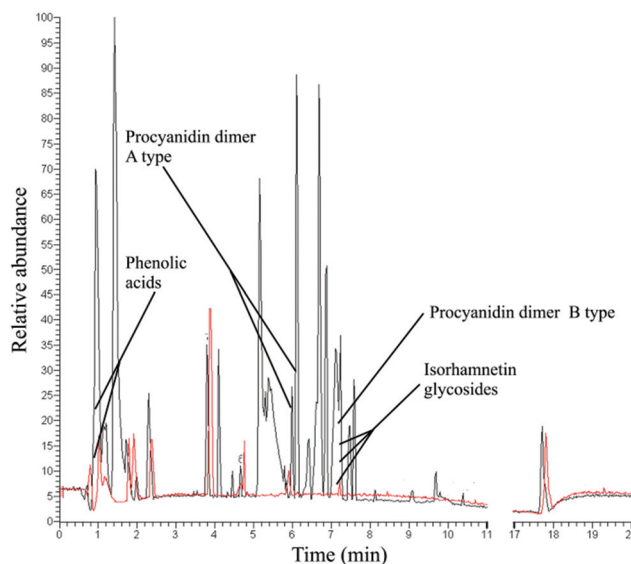

Fig. 5 UHPLC chromatograms of peanut polyphenols (black) and laccase-treated peanut polyphenols (red). A difference in the abundance of phenolics is clearly visible. The experiment was performed in triplicate. Sample spectra are shown.
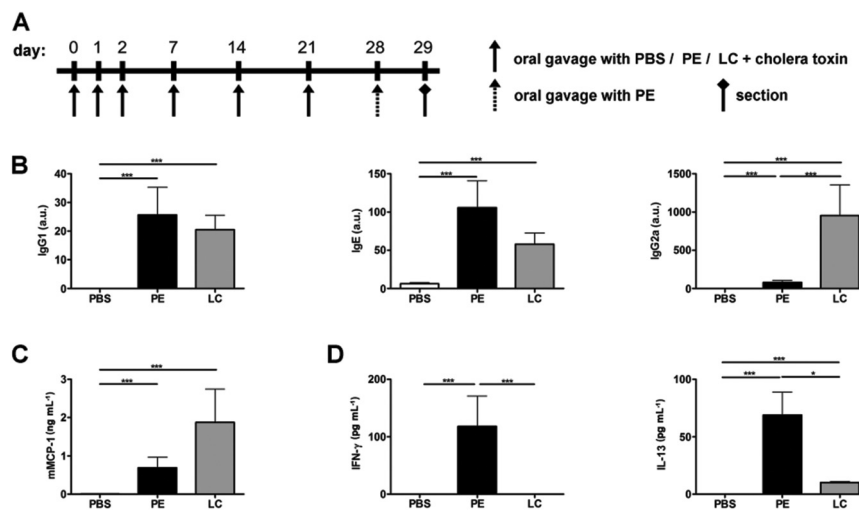

Fig. 4 Ability of the cross-linked material to induce allergic sensitization: A – sensitization protocol; B – IgG1, IgE, and IgG2a concentrations (a.u.) on day 29; C – mMCP-1 (ng mL^{-1}) in serum; D – IFN-γ and IL-13 measured in spleen-cells culture (pg mL^{-1}). *$p < 0.05$, ***$p < 0.001$. All measurements were performed in triplicate. PBS – phosphate buffered saline, PE – untreated peanut extract, LC – laccase-treated peanut extract.

Table 2 Relative amounts of phenolic compounds identified in laccase-treated peanut extract in comparison with untreated peanut-extract. Results are expressed as percentage (%) of a peak area of the corresponding compound found in untreated extract. Data acquisition was performed in negative mode. ND – not detected

m/z	Retention time (min)	Identified phenolic compound	Relative amounts of phenolics (%)
137.04	0.95	*p*-Hydroxybenzoic acid	1.32
153.02	1.47	Protocatechuic acid	ND
163.04	1.35	*p*-Coumaric acid	1.92
167.07	1.44	Vanillic acid	9.01
169.04	0.95	Gallic acid	ND
193.08	1.22	Ferrulic acid	5.08
179.06	1.26	Caffeic acid	0–7.11
187.10	4.71	Coumarine derivative	0.61
289.08	5.17	Catechin/epicatechin isomers	0.17
293.11	5.34	Catechin/epicatechin isomers	1.21
295.02	3.13	Catechin/epicatechin isomers	ND
329.23	7.59	Isorhamnetin-3-methoxy derivative	0.72
329.23	7.32	Isorhamnetin-3-methoxy derivative	ND
353.20	2.21	Chlorogenic acid	ND
457.08	4.10	Epigallocatechingallate	ND
441.08	4.37	Epicatechingallate isomers	ND
441.08	4.45	Epicatechingallate isomers	ND
441.08	4.91	Epicatechingallate isomers	ND
441.08	5.12	Epicatechingallate isomers	ND
477.16	6.18	Isorhamnetin-glucoside derivative	5.17
575.20	6.09	Procyanidin dimer A-type	0.06
575.20	6.18	Procyanidin dimer A-type	0.08
579.17	7.09	Procyanidin dimer B-type	0.51
579.17	7.15	Procyanidin dimer B-type	ND
587.23	8.32	Flavonoid derivative	0.13
619.24	4.10	Epigallocatechingallate glucoside	ND

HPLC-MS/MS analysis of phenolic compounds of peanut extract revealed the presence of A- and B-type procyanidins and other flavonoid compounds, as well as phenolic acids (Fig. 5 and Table 2).

After the enzymatic reaction, HPLC-MS/MS analysis showed that the relative amounts of phenolics were significantly reduced. Isorhamnetin derivatives and a procyanidin B-type dimer could be observed in the laccase-treated peanut extract, with additional peaks ascribed to unidentified compounds. Most phenolic compounds (catechin/epicatechin isomers, epicatechin-gallate, procyanidin A-type dimer) were not detected in the laccase-treated sample (Table 2). The concentrations of selected compounds (gallic acid, vanillic acid, *p*-coumaric acid, ferullic acid) were determined to be more than 10 times higher in the control compared to the laccase-treated sample (Table 3).

Table 3 Concentrations of selected phenolic acids in peanut extract and laccase-treated peanut extract. Experiments were performed in triplicate. ND – not detected

Phenolic compound	Concentration of phenolics ± SD (mg kg^{-1})	
	Peanut extract	Laccase-treated peanut extract
Gallic acid	50.38 ± 1.24	ND ± –
Vanillic acid	115.05 ± 5.71	10.37 ± 0.56
Ferrulic acid	99.83 ± 0.38	5.08 ± 0.43
p-Coumaric acid	5552.90 ± 89.47	106.76 ± 4.69

4. Discussion

By using laccase-cross-linked peanut proteins, we have investigated the effect of cross-linking on the protein structure, enterocyte adsorption and capacity to induce allergic sensitization in a mouse model of allergy to peanuts. Lacasses are enzymes which may use intrinsic polyphenols as auxiliary substrates for the cross-linking reactions; therefore the composition of peanut polyphenols was analyzed before and after the enzymatic reaction.

First, we have demonstrated that the laccase used in this study efficiently cross-linked peanut proteins, by using only intrinsic peanut polyphenols. Larger aggregates, with molecular weights of 500–800 kDa and over 1000 kDa were obtained by the action of laccase, comparable to the action of transglutaminase or tyrosinases on peanut proteins.[9,13] For instance, treatment of peanut proteins with mushroom tyrosinase resulted in aggregates of sizes between 500–800 kDa, while *T. reesei* tyrosinase yielded cross-links of around 500 kDa in size.[9] Transglutaminase treatment also only partially cross-linked peanut proteins.[13] The enhanced cross-linking efficacy by laccase may be due to efficient covalent incorporation of peanut phenolics and creation of linkers between protein molecules.[32]

Far UV and near UV CD spectroscopy analyses revealed that cross-linking by laccase mostly affected the tertiary structure of peanut proteins. The secondary structure of proteins and the overall protein fold were not significantly disturbed by

enzymatic action. Reduced binding of specific antibodies implies a significant change in the tertiary structure of major allergenic peanut proteins Ara h 3 and Ara h 6. Ara h 2 showed the least propensity to be modified by laccase. Being the most dominant allergen in peanut[33] and the major elicitors of anaphylaxis that account for the majority of effector activity in a crude peanut extract,[34] this may explain the marginal effects of the applied cross-linking treatment on the allergenicity of peanut (ESI, Fig. S2 and S3†). Clare *et al.* demonstrated that Ara h 2 remained essentially intact even after 2–3 h of treatment with transglutaminase,[13] which suggest that a compact fold of Ara h 2 makes this protein a difficult substrate for various cross-linking enzymes, not only laccase.

Significant changes in size, shape and hydrophobicity of proteins induced by cross-linking can be relevant for the processes of adsorption and uptake by enterocytes. Caco-2 cell monolayers are an *in vitro* system often used as a model for the small intestinal epithelium, including transport studies of different food components.[35] Bioavailability experiments showed that the allergens from cross-linked products passed through the Caco-2 cell monolayer more efficiently. The concentration of peanut allergen Ara h 2 passing the layer increased up to 70 fold following the treatment by laccase. Heat-induced aggregation of Ara h 2 positively affected adsorption and uptake of this protein by the Caco-2 cells.[36] Bioavailability testing of a tyrosinase-treated peanut extract showed similar results.[9]

Processes that affect the transport of globular proteins can lead to changes in food allergy and tolerance mechanisms for these allergens. For instance, it has been shown that aggregation of globular whey proteins, beta-lactoglobulin and alpha-lactalbumin, by pasteurization resulted in reduced transport in Caco-2 cell monolayer and promoted food allergy *in vivo*.[37] Laccase cross-linking of beta-lactoglobulin, influenced food allergy development *in vivo*, increased sensitizing capacity and significantly reduced transcytosis of the cross-linked protein in a Caco-2 model system.[8]

This report demonstrates that laccase treatment of peanut proteins did not increase the *in vivo* allergenicity towards peanut proteins. However, the laccase treatment of peanut extract resulted in a modulated immune response in animals by increasing IgG2a responses *in vivo*.

In mice, as well as in humans, the Th2 (T helper type 2) cell immune response is a hallmark of hypersensitivity disorders and characterized by IgE and IgG1 production, as well as production of allergy-promoting cytokines, such as IL-13, while the Th1 immune response is characterized by increased production of IgG2a antibodies in mice and Th1-type cytokines, such as IFN-γ.[38]

An increase in IgG2a production implies that the balance of the immune response is pointed towards the Th1 response.

This result is further supported by the downregulation of IL-13. IL-13 is a cytokine secreted by (amongst others) Th2-type lymphocytes and is a mediator of allergic inflammation and disease. IL-13 activates the same signal transduction pathways as IL-4 and induces IgE production.[39]

A recent study showed that the three-dimensional structure has a significant impact on the antibodies raised for both systemic and orally administered allergens. A remarkable difference in the antibody binding patterns against linear and conformational epitopes was seen between the allergens, indicating that the structural characteristics of proteins may heavily affect the induced antibody response.[40] We have demonstrated that laccase influences the structure of peanut proteins, which likely contributes to *in vivo* immunomodulatory features of the laccase-cross-linked peanut protein extract.

In addition, peanuts are known to be a rich source of resveratrol, quercetin, epigallocatechin 3-gallate and different procyanidins,[41–43] which are important components of a whole peanut extract. Many of these phenolic compounds of peanut can be the substrates/mediators of enzymatic oxidation by laccase and subject to polymerization reactions.[44]

The polymerized polyphenols may also precipitate proteins from complex mixtures.[45] As the binding affinity between proteins and polyphenols increases with the size of polyphenols[46] the polymerization reaction will eventually deplete polyphenols from the extract, as has been observed in our study.

The presence of the peanut food matrix has a profound effect on the *in vivo* immune response to peanut proteins and studies have demonstrated that the purified peanut allergens possess little intrinsic immune-stimulating capacity in contrast to a whole peanut extract.[30] We suppose that the products of laccase action on peanut matrix polyphenols most likely contribute to the immunomodulatory effects, since no similar findings are shown if the proteins were cross-linked by enzymes which do not use polyphenols as auxiliary substrates.[9]

5.   Conclusions

Laccase cross-linking disturbs the peanut protein tertiary structure and affects the peanut polyphenol composition. The extensively cross-linked proteins have unchanged potency to induce allergic sensitization, but certain immunomodulatory changes were observed *in vivo*. The animals sensitized to laccase-cross-linked peanut proteins show increased serum IgG2a levels and diminished production of IL-13 by spleen cells in comparison with the animals sensitized to untreated peanut extract.

Conflict of interest

The authors have declared no conflict of interest.

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