Supplementary data for the article:

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Material and Methods

S1 *In vitro* digestion

Digestion by pepsin (Sigma-Aldrich, Munich, Germany) was performed at a ratio of 10 U of pepsin activity per 1 microgram of protein at pH 1.20.[1] Samples were incubated at 37 °C for 1, 5, 15, 45 and 180 min and aliquots of 100 µL were taken and the reaction was stopped by adding 30 µL of 2 M sodium bicarbonate, followed by adding 33 µL of reducing Laemmli buffer and boiling for 5 min at 95 °C. Controls had no pepsin added. After digestion, the samples (50 µL of each) were analysed in duplicate on 16% SDS-polyacrylamide gels, using Tris-glycine buffer system.[2] The voltage was kept at 100 V until the dye front reached separating gel, and then was increased to 200 V and kept constant.[2] The total run time was 2 h.

S2 Basophil activation

Basophil activation tests were performed as described previously.[3] Briefly, heparinized blood samples were taken from 4 peanut-allergic patients. The study was approved by the Ethical Committee of the University Children's Hospital, Belgrade, Serbia (Approval number: 017/6-990/66). All participants gave informed consent to participate in the study.
For activation assays, blood aliquots (100 µL) were incubated with allergens at four different concentrations (range 0.05-50 µg mL^{-1}), anti-IgE antibody as a positive control (10 µg mL^{-1}), or PBS as a negative control, for 15 min (37 °C). After incubation, cells were washed in PBS and then incubated with 10 µL of phycoerythrin-labelled CD203c monoclonal antibody 97A6 (Immunotech, Marseille, France) and FITC-labelled CD63 monoclonal antibody (Immunotech, Marseille, France) for 15 min at room temperature. Thereafter, samples were subjected to erythrocyte lysis with 2.0 mL of FACS lysing solution (BD Biosciences, San Jose, CA, USA). Cells were then washed, resuspended in PBS, and analyzed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The basophil marker CD203c was used to set a basophil gate including only CD203c+ cells. The collected cells were further analyzed for granule release using an anti-CD63 antibody. The extent of basophil activation (BA) was calculated as the percentage of CD63+ events among the gated basophils (CD63+/CD203c+ cells).

S3 Human IgE binding

Human sera were taken from patients of both genders, aged 19-45, 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 (Phadia Diagnostics, Uppsala, Sweden). Levels were considered positive if above 0.35 kAU L^{-1}. Pooled sera used in immunoblot experiments were obtained from 7 patients and had an average of 37.00 kAU L^{-1}. Pooled sera used in ELISA inhibition experiments were obtained from 14 patients and had an average of 30.80 kAU L^{-1}. Prior to use all sera were centrifuged for 15 min at 10,000 x g. All the sera used in IgE binding experiments were obtained at University Children's Hospital, Belgrade, Serbia (Approval: 017/6-990/66).

S3.1 Immunoblot

To study the immunoreactivity of peanut allergenic proteins, 12% SDS-PAGE gels were prepared[2] and the separated protein cross-links were transferred to nitrocellulose membrane (Roti-NC, Carl Roth GmbH, Munich, Germany), according to Towbin *et al.*[4] Electrotransfer was performed at 1.5 mA cm^{-2} of membrane surface for 45 min.
After electrotransfer, membrane was blocked with 1.00% BSA in TTBS for 3 h and incubated with a pool of peanut allergic patient sera (diluted 1:5 (v:v) with TTBS). Bound IgE was detected using mouse anti-human IgE antibodies (Sigma–Aldrich, Munich, Germany), labelled with alkaline phosphatase, diluted in TTBS supplemented with 0.10% BSA in appropriate dilutions (1:2,000). The binding patterns were visualized with a substrate solution consisting of 1.5 mg BCIP (5-bromo-4-chloro-3-indolyl phosphate) and 3.0 mg NBT (nitrobluetetrazolium) in 10.0 mL of 100 mM Tris buffer, containing 150 mM NaCl, and 5 mM $MgCl_2$, pH 9.60. Developed membrane was scanned with Epson Perfection V330 scanner (Epson, Tokyo, Japan) at 600 dpi resolution in grayscale mode.

S3.2 Inhibition ELISA for determination of IgE binding potency of cross-linked peanut proteins

Materials for inhibitions were prepared at 10 fold serial dilutions and preincubated for 1 h with the aliquots of the human sera pool (final dilution: 60 fold).

Costar ELISA plates (Corning, San Diego, CA, USA) were coated overnight with 100 µL of peanut proteins in PBS (concentration: 10.0 mg mL^{-1}). After washing with TPBS, blocking with 1.00% BSA in TPBS for 1 h and a washing step with TPBS, preincubated sera (100 µL) were applied onto plates and incubated for 2 h. Subsequently, plates were washed and incubated with goat-anti-human IgE-HRP conjugated

antibodies (Pharmingen, San Diego, CA, USA) for 1 h. ELISA was visualized with 100 µL TMB substrate solution (0.1 mg mL^{-1}). The reaction was stopped after 15 min with 50 µL 1 M H_2SO_4 and optical density at 450 nm was measured.
Percentage of inhibition (% of inhibition) was expressed as (A_{450} noninhibited - A_{450} inhibited) / A_{450} noninhibited × 100%.

Results

Susceptibility of obtained material to pepsinolysis

Pepsinolysis of the untreated and laccase-treated peanut extracts was performed to investigate effects of the laccase treatment on proteins' stability in the acidic environment and resistance to action of proteases. Unlike the untreated peanut extract, laccase-treated peanut proteins showed prolonged survival of high molecular weight aggregates for over 3 h. (Figure S1)

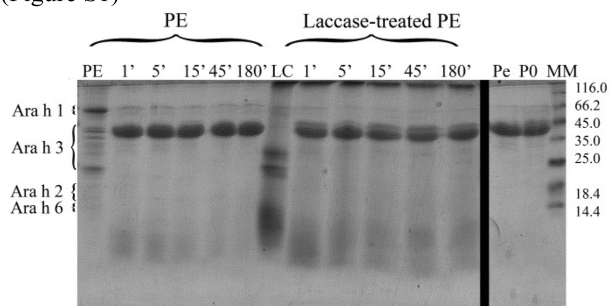

Figure S1: Pepsinolysis of peanut extract and laccase-treated peanut extract: PE, LC - peanut extract and laccase-treated peanut extract, respectively, 1', 5', 15', 45', 180' - duration of pepsinolysis (in min). Pe and P0 – pepsin only at 180 and 0 min of digestion, MM – molecular weight markers (in kDa). Experiment was performed in duplicate, a sample gel is shown. All samples are analysed in the same gel. Vertical black line indicates discontinuation in the gel.

Human IgE binding to laccase-modified peanut proteins

Effects of modifications by laccase on binding of peanut proteins to human IgE were investigated by basophil activation assays, immunoblot and inhibition ELISA.

Basophils were stimulated with laccase-treated material in the similar manner as with unmodified peanut extract (Figure S2).

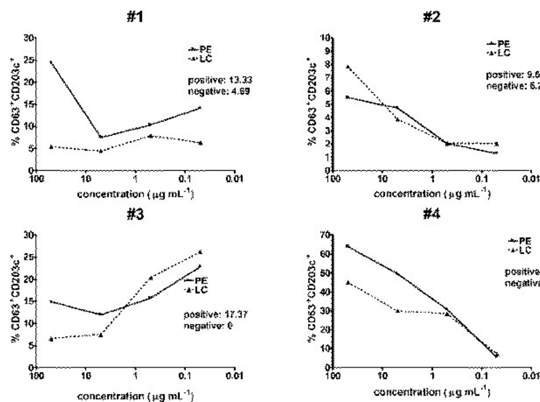

Figure S2: Four peanut-allergic patients' basophil activation with peanut extract (PE) and laccase-treated peanut extract (LC).

In order to quantify the effects of cross-linking on allergenicity of peanut proteins, inhibition assays by ELISA were performed with pooled peanut allergic patient sera and IC_{50} was determined (Figure S3B). The obtained IC_{50} values for untreated PE were within the same order of magnitude as cross-linked proteins which implies that no significant modification of IgE binding epitopes of peanut proteins occurred when peanut proteins were modified by the cross-linking enzyme. An immunoblot using pool of peanut-allergic patients' sera and detection of bound IgE (Figure S3A) appeared to show a certain degree of reduction of IgE binding, but these results should be interpreted with caution. As the protein bands were smeared due to cross-linking, it was not possible to directly compare band intensities between samples. A 66 kDa band (presumably Ara h 1) was visible in both samples. A reactive band could be observed at the top of the gel in the laccase-treated peanut extract, indicating that IgE binding was preserved in the obtained cross-links of high molecular weight.

In line with this, linear epitopes in peanut allergic subjects tested seemed to be more important than conformational.

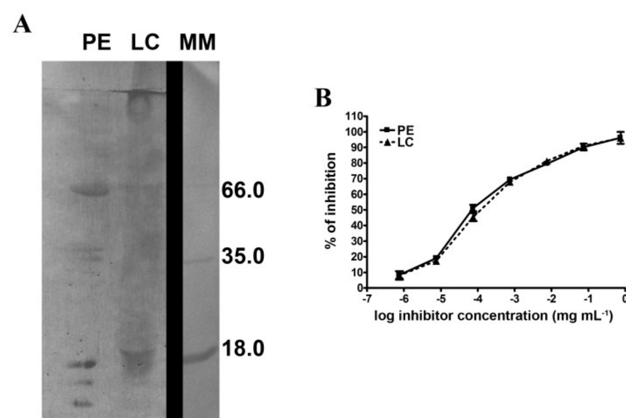

Figure S3: Human IgE binding to peanut extract (PE) and laccase-treated peanut extract (LC). A – immunoblot. MM – molecular weight markers (in kDa); B – ELISA inhibition.

References:

1. K. Thomas, M. Aalbers, G. A. Bannon, M. Bartels, R. J. Dearman, D. J. Esdaile, T. J. Fu, C. M. Glatt, N. Hadfield, C. Hatzos, S. L. Hefle, J. R. Heylings, R. E. Goodman, B. Henry, C. Herouet, M. Holsapple, G. S. Ladics, T. D. Landry, S. C. MacIntosh, E. A. Rice, L. S. Privalle, H. Y. Steiner, R. Teshima, R. Van Ree, M. Woolhiser and J. Zawodny, *Regul. Toxicol. Pharmacol.*, 2004, **39**, 87-98.
2. E. Harlow and D. Lane, *Antibodies: a laboratory manual*, Cold Spring Harbor Laboratory, New York, 1988.
3. D. Stanic, E. Monogioudi, E. Dilek, J. Radosavljevic, M. Atanaskovic-Markovic, O. Vuckovic, L. Raija, M. Mattinen, J. Buchert and T. Cirkovic Velickovic, *Mol. Nutr. Food Res.*, 2010, **54**, 1273-1284.
4. H. Towbin, T. Staehelin and J. Gordon, *Proc Natl Acad Sci U S A*, 1979, **76**, 4350-4354.

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