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Potential changes in the allergenicity of three forms of peanut after thermal processing

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ABSTRACT

This study aimed to analyze the influence of thermal processing on the IgE binding properties of three forms of peanut, its effects in the content of individual allergens and IgE cross-linking capacity in effector cells of allergy. Three forms of peanut were selected and subjected to thermal processing. Immunoreactivity was evaluated by means of immunoblot or ELISA inhibition assay. Specific antibodies were used to identify changes in the content of the main allergens in peanut samples. The ability of treated peanut to cross-link IgE was evaluated in a basophil activation assay and Skin Prick Testing (SPT). The results showed that thermal/pressure treatments at specific conditions had the capacity to decrease IgE binding properties of protein extracts from peanut. This effect went along with an altered capacity to activate basophils sensitized with IgE from patients with peanut allergy and the wheal size in SPT.

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1. Introduction

Peanut allergy is one of the most severe food allergies; its prevalence has risen during past decades in western countries (Grundy, Matthews, Bateman, Dean, & Arshad, 2002; Sicherer & Sampson, 2007). Differences in the processing of peanuts are suggested to influence the allergenic sensitization to this legume. The chemical modifications that occur in peanuts during roasting, in which a reaction between free amino groups on proteins and reducing sugars is produced (Maillard's reaction), seem to affect the allergenicity of peanut proteins. It has been demonstrated that roasted peanut extracts bind IgE from patients with peanut allergy more efficiently than raw peanuts (Maleki, Chung, Champagne, & Raufman, 2000). Boiling, in contrast, seems to reduce *in vitro* IgE reactivity to the boiled peanut proteins. This phenomenon has been recently correlated with clinical reactivity (Turner et al., 2014).

Processing methods applied during manufacturing by industry or post-manufacturing by consumers to improve food characteristics and properties have the potential to modify food allergenicity. The molecular basis of these allergenic alterations can include disruption of conformational epitopes, exposure of cryptic epitopes by denaturation of the native allergenic structures and formation of new epitopes due to chemical modifications. The extent of these modifications depends on factors, such as biochemical properties of the allergens, food matrix or processing conditions (Besler, Steinhart, & Paschke, 2001). While it is clear from the scientific literature that certain thermal or enzymatic treatments have the potential to decrease IgE binding capacity *in vitro* as it has been demonstrated for certain nuts and legumes (Cabanillas et al., 2012; Clemente, Vioque, Sanchez-Vioque, Pedroche, & Millán, 1999), the reduced IgE binding capacity of processed food sometimes does not correlate with a decreased capacity of treated food to cross-link IgE on effector cells, such as basophils or mast cells (Shi et al., 2013). Importantly, it has been shown that peptides of 30–50 amino acids containing two epitopes for IgE can cross-link IgE on effector cells (Fu, 2002). Therefore, an extensive protein fragmentation seems to be necessary to ensure a reduced allergenicity. In peptide immunotherapy, small peptides with no capacity for IgE cross-linking on mast cells or basophils but with the potential for CD4+ T cell stimulation are used. Therefore, processing of food with the capacity to generate peptides with decreased IgE binding capacity and tolerogenic effects would be a potential strategy for oral

Abbreviations: BCA, Bicinchoninic acid assay; DBPCFC, double-blind placebo-controlled food challenge; FEIA, Fluorenzymeimmunoassay; HRP, horseradish peroxidase; PVDF, polyvinylidene difluoride; SPT, Skin Prick Testing; TBS, Tris-buffered saline; TBST, TBS plus 0.5% Tween-20; TMB, tetramethylbenzidine.

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immunotherapy. Clinical trials have demonstrated that a high percentage of children, with milk and egg allergies, tolerated heated products with milk or egg. This clinical effect goes along with increased levels of specific IgG4 antibodies (Lemon-Mulé et al., 2008; Nowak-Wegrzyn et al., 2008).

We have previously demonstrated that food processing, such as treatments based on pressure and heat, seem to influence the IgE binding capacity of nut proteins (Cabanillas et al., 2012). In the present study we aimed to further analyze the effect of thermal processing on the IgE binding properties of three forms of peanut, and to investigate if changes in IgE binding capacity of peanut proteins due to processing go along with an altered capacity to cross-link IgE on the effector cells of allergy, such as basophils.

2. Materials and methods

2.1. Patients and sera

Sera from 8 Spanish patients with peanut allergies, confirmed on the basis of either a history of recent documented severe anaphylaxis after peanut ingestion or a positive double-blind placebo-controlled food challenge (DBPCFC) with peanut, were used for the *in vitro* experiments. Subjects had specific serum IgE levels to peanut ranging from 0.47 to 7.43 kU/l (median = 3.5 kU/l) as quantified by the fluorescent enzyme immunoassay (CAP-FEIA system, Phadia, Uppsala, Sweden). After informed consent, 7 additional Spanish patients with confirmed clinical allergies to peanuts and with specific serum IgE levels to peanut ranging from 0.36 to 6.7 kU/l (median = 1.26 kU/l) underwent SPT with untreated and treated peanuts according to standard methods (Mailing, 1993). Two healthy subjects were tested with the same samples as a control group. The study was approved by the Ethics Committee of the Hospital Universitario 12 de Octubre, Madrid, Spain (Permission No. 0312150129).

2.2. Plant material and processing treatments

Three forms of peanut: raw, fried and roasted (*Arachis hypogaea*, variety Virginia) were purchased at Aperitivos Medina SL (Spain). Seeds were immersed in distilled water (1:5 w/v) and treated using a tabletop autoclave (CertoClav Multicontrol IPX4, Traun, Austria) (AU treatments) at 121 °C, 1.18 atm, for 15 min (AU1 treatment) or 30 min (AU2 treatment) and at 138 °C, 2.56 atm for 15 min (AU3 treatment) or 30 min (AU4 treatment).

Additionally, raw peanut seeds were boiled in water (1:5 w/v) at 100 °C, for 60 min. The seeds were then milled to pass through a 1 mm sieve (Tecator, Cylotec 1093, Höganäs, Sweden) and the resulting meal was defatted with n-hexane (34 ml/g of flour) for 4 h, shaken, and air-dried after filtration of the n-hexane. Protein extraction was carried out twice using a solution of 50 mM Tris–HCl (pH 8.0) plus 500 mM NaCl at 1:10 ratio for 1 h, at 4 °C, with stirring. After centrifugation at 27,000*g*, 20 min, 4 °C, the supernatants were dialyzed against water using a membrane with a cut-off point of 3.5 kDa during 48 h at 4 °C and then the supernatants were freeze-dried. The bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL USA) was used for protein quantitation. The nitrogen contents of the samples were determined by LECO analysis according to standard procedures based on Dumas method (AOAC, 2000). The total protein content was calculated as N × 5.45 (AOAC, 2000).

2.3. Protein electrophoresis and Ara h 1, Ara h 2 and Ara h 6 detection in western blot and dot blot

SDS–PAGE was performed according to Laemmli (1970). Untreated and treated peanut samples (20 μg protein per lane) were mixed with Laemmli sample buffer and β-mercaptoethanol heated at 95 °C for 10 min, and electrophoresed in a 12% Tris–HCl gel. Proteins were visualized with Coomassie Brilliant Blue (Bio-Rad, Hercules, CA, USA).

For western blot, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). Blocking was carried out for 1 h, at room temperature in Tris-buffered saline (TBS) containing 0.5% Tween-20 (TBST) and 5% non-fat milk (blocking solution). IgG mouse anti-Ara h 1 (stock: 2.7 mg/ml), anti-Ara h 2 (stock: 2 mg/ml) and anti-Ara h 6 (stock: 2 mg/ml) monoclonal antibodies (clones 2C12, 1C4 and 3B8, respectively. Indoor Biotechnologies, Charlottesville, VA, USA) were diluted in blocking solution (1:5000 for anti-Ara h 1 and anti-Ara h 2; 1:2500 for anti-Ara h 6) and incubated with the PVDF membranes for 2 h (for anti-Ara h 1 and anti-Ara h 2) or overnight (for anti-Ara h 6). Membranes were washed and then treated with horseradish peroxidase (HRP) conjugated goat anti mouse antibody (stock: 0.4 mg/ml. Used at 1:5000) (Santa Cruz Biotechnology, Dallas, Texas, USA) diluted in TBST containing 2.5% non-fat milk. Detection was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (GE Healthcare Life Sciences, Buckinghamshire, UK). The signal was measured using ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare Life Sciences, Buckinghamshire, UK). Dot blot was performed by applying samples (10 μg protein per dot) on PVDF membranes and proceeded as described above for the western blot with anti-Ara h 1, anti-Ara h 2 and anti-Ara h 6 antibodies.

In order to compare the electrophoretic patterns of soluble protein fractions with those of total protein from defatted flours of untreated and treated peanuts, extraction of total protein was also carried out. Flours were directly solubilized in electrophoresis SDS sample buffer as previously described (Cabanillas et al., 2014). Twenty micrograms of total protein from each sample (calculated from LECO analysis) was electrophoresed.

2.4. ELISA

Polystyrene microtiter plates (BD Falcon 353279, Heidelberg, Germany) were coated with 100 μl/well of untreated or treated raw, fried or roasted peanut protein extracts (50 μg/ml in PBS) and incubated overnight at 4 °C. Wells were washed with PBS containing 0.5% Tween-20 and blocked with PBS containing 3% non-fat milk plus 0.1% Tween-20. Plates were incubated with IgG mouse anti-Ara h 1, anti-Ara h 2, or anti-Ara h 6 monoclonal antibodies for 2 h, washed and incubated with HRP-conjugated goat anti mouse antibody for 1 h. All antibodies were used at the same dilution factors as in the western blot. After washing, the peroxidase reaction was developed with a tetramethylbenzidine (TMB) substrate (substrate reagent pack, DY999, R&D Systems, Inc., Minneapolis, MN, USA). The reaction was stopped with 2 N H_2SO_4, and the OD was measured at 450 nm. Wells coated with blocking solution instead of peanut protein samples and incubated directly with the primary and secondary antibodies were used as negative controls. All the tests were performed in duplicate. The formula: mean [OD] + 3 × SD was calculated for the negative controls to establish the cut-off point of positivity.

2.5. ELISA inhibition assays

Polystyrene microtiter plates (BD Falcon 353279, Heidelberg, Germany) were coated with 100 μl/well of raw, fried or roasted peanut protein extracts (250 μg/ml in PBS) and incubated overnight at 4 °C. In parallel, a serum pool from the peanut allergic patients (1:10) was preincubated with untreated or treated raw, fried or roasted peanut protein extracts (inhibitors) (final concentrations: 100, 10 and 1 μg/ml) overnight at 4 °C with stirring.

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