

ORIGINAL ARTICLE

Effect of roasting on the allergenicity of major peanut allergens Ara h 1 and Ara h 2/6: the necessity of degranulation assays

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Summary

Background Peanuts are often consumed after roasting, a process that alters the three-dimensional structure of allergens and leads to Maillard modification. Such changes are likely to affect their allergenicity.

Objective We aimed to establish the effect of thermal treatment mimicking the roasting process on the allergenicity of Ara h 1 and a mix of 2S albumins from peanut (Ara h 2/6).

Methods Ara h 1 and Ara h 2/6 were purified from raw peanuts and heated in a dry form for 20 min at 145 °C in the presence (R+g) or absence (R–g) of glucose, and soluble proteins were then extracted. Sera obtained from 12 well-characterized peanut-allergic patients were used to assess the IgE binding and degranulation capacities of the allergens.

Results Extensive heating at low moisture resulted in the hydrolysis of both Ara h 1 and Ara h 2/6. However, in contrast to Ara h 2/6, soluble R+g Ara h 1 formed large aggregates.

Although the IgE-binding capacity of R+g and R–g Ara h 1 was decreased 9000- and 3.6-fold, respectively, compared with native Ara h 1, their capacity to elicit mediator release was increased. Conversely, both the IgE-binding capacity and the degranulation capacity of R–g Ara h 2/6 were 600–700-fold lower compared with the native form, although the presence of glucose during heating significantly moderated these losses.

Conclusions and Clinical Relevance Extensive heating reduced the degranulation capacity of Ara h 2/6 but significantly increased the degranulation capacity of Ara h 1. This observation can have important ramifications for component-resolved approaches for diagnosis and demonstrates the importance of investigating the degranulation capacity in addition to IgE reactivity when assessing the effects of food processing on the allergenicity of proteins.

Keywords allergenicity, Ara h 1, Ara h 2/6, glycation, histamine release, IgE binding, peanut allergy, RBL, roasting, thermal processing

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Introduction

Peanut allergy is a common food allergy in the United States and in European countries, affecting around 1% of the population [1, 2]. In some studies, the prevalence was found to be much higher, with approximately 10% of 8-year-old children in the United Kingdom being sensitized to peanut, of whom 2% have a clinical peanut allergy [3].

In addition, the prevalence of peanut allergy in children appears to be increasing [2], which contributes to a growing global concern, particularly given the severity of peanut-allergic reactions.

Recent studies indicate that peanut-allergic patients are most frequently sensitized to Ara h 2 and Ara h 6, followed by Ara h 1, Ara h 3 and Ara h 7 [4, 5]. Ara h 6 and Ara h 7 show around 55% and 40% amino acid

sequence homology with Ara h 2, respectively [6, 7], suggesting that these three proteins could share a number of identical epitopes [6, 8]. However, epitopes specific to Ara h 2 and Ara h 6 also exist [4]. Ara h 2, Ara h 6 and Ara h 7 all belong to the family of the 2S albumin storage proteins and have a molecular weight between 16 and 20 kDa [6]. Ara h 1 is a vicilin-like 7S seed storage globulin, also known as conarachin. Ara h 1, comprising 63.5 kDa subunits that assemble naturally into trimers of approximately 180 kDa, often aggregates, forming multimers of up to 600–700 kDa [9–11]. Twenty-three linear IgE epitopes have been identified within Ara h 1, mainly in the areas of the subunit–subunit contacts that are protected from protease degradation [12–14].

Food processing can potentially destroy or conversely create linear and conformational IgE-binding epitopes, establish the exposure of formerly hidden antigenic sites and change the susceptibility to digestion [15]. These changes may affect allergic sensitization to food proteins and elicitation of allergic reactions in sensitized individuals, thereby impacting on the allergenic potential of the food proteins. Important modifications that food proteins may undergo are observed as a consequence of the Maillard reaction, which involves the reaction of free amino groups on proteins (particularly lysine residues) with reducing sugars, thereby inducing flavour and colour of processed foods including peanuts [16, 17]. The extent of glycation depends on the temperature, pH, water activity, duration of the heating and the concentration of the reducing sugars present [18]. While the Maillard reaction can affect the allergenicity of peanut proteins, this has not been conclusively demonstrated and depends on the type of protein and the conditions under which the Maillard reaction occurred. In addition, the type and amount of carbohydrate used and other matrix compounds present during heating also affect allergenicity [16, 17, 19–23].

The effect of processing on the allergenicity of proteins has been assessed by determining the IgE-binding capacity using immunoassays and/or immunoblotting. However, the impact of food processing on the cross-linking and degranulation capacity is important, as processing-induced modifications are likely to affect the availability of the multiple epitopes required for effective cross-linking of cell surface-bound IgE. In the current study, the effects of processing were also assessed using two cellular mediator release assays (MRAs) to determine the allergen cross-linking capacity of IgE antibodies bound to FcεRI of mast cells/basophils. Roasting induces an array of protein modifications due to interaction with the food matrix, affecting the purification efficacy of the modified allergens. Therefore, the allergens were first purified from untreated peanut, lyophilized and then subjected to a model thermal treatment mimicking the roasting process.

Materials and methods

Patients

Peanut-allergic patients from the Allergology Practice Arnhem (APA, the Netherlands) were approached to participate via an invitation letter. Written informed consent was obtained before sample collection and the experiments performed were approved by the local ethical committee (Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen). The population studied included 12 peanut-allergic patients. All patients had a well-documented peanut allergy based on an extensive medical history, physical examination and objective clinical manifestations observed after peanut ingestion. Grading of food-induced anaphylaxis was according to the severity of clinical symptoms [24, 25]. From all patients, except one, a skin prick test with a commercial peanut extract (ALK-Abelló, Nieuwegein, the Netherlands) was performed and peanut-specific IgE levels were determined using ImmunoCAP (Phadia AB, Uppsala, Sweden).

Isolation of Ara h 1 and Ara h 2/6

Native Ara h 1 (N-Ara h 1) was purified from redskin-type raw peanuts (obtained from local suppliers, Norwich, UK) as described by Marsh *et al.* [26]. The purification is based on affinity chromatography using concanavalin A-Sepharose, followed by gel permeation chromatography using a Superdex Preparative Grade S200 column (GE Healthcare, Buckinghamshire, UK). Protein preparations were adjudged >99% pure by SDS-PAGE.

Native 2S albumins containing both Ara h 2 and Ara h 6 (N-Ara h 2/6) were purified from unroasted redskin-type peanuts as described previously and allergen identity was confirmed by in gel-trypsin digestion and MALDI-TOF mass spectrometry [27]. This purification involved ammonium sulphate fractionation and gel-filtration chromatography, avoiding the use of denaturing conditions such as reverse-phase HPLC. Purified native proteins were stored at –20 °C before use.

Roasting treatment of the purified allergens

Blanched kernels of peanuts are subjected to oil roasting [28], a process that results in extensive, but not complete, denaturation of peanut kernel proteins. In order to mimic this process, purified proteins were freeze dried to reduce the moisture content to about 4–5% [29], which corresponds to the moisture content of blanched peanuts. The inclusion of sugar in the samples before freeze-drying resulted in a further reduction of the moisture content, to about 1.5–3% [29].

Purified N-Ara h 1 in 32.5 mM phosphate buffer (pH 7.6) containing 100 mM NaCl and purified N-Ara h 2/6 in

25 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and 0.02% (w/v) NaN₃ were divided into three batches. One batch of the protein was kept untreated (native form). One batch was mixed with glucose (Sigma-Aldrich, Steinheim, Germany) in a ratio of 1 : 2 (protein : glucose, w/w) in phosphate-buffered saline (PBS). The batch with glucose and one batch without glucose were placed in glass bottles, frozen at -70°C and lyophilized. Lyophilized samples were then heated in a forced-convection Chamber of Thermal Research (Wamed, KBC-65W) under conditions chosen according to Saklar et al. [30]. After establishing a steady temperature of 145°C , the proteins were heated for 20 min and then samples were allowed to cool to ambient temperature. Samples were dissolved in Milli-Q water, centrifuged ($5000\times g$, 15 min, 4°C) and the supernatant was collected and ultrafiltered by Amicon Ultra-15 centrifugal filters [10 000 nominal molecular weight limit (NMWL) for Ara h 1 and 5000 NMWL for Ara h 2/6] (Millipore, Billerica, MA, USA) against PBS for several rounds to remove the residual glucose, the hydrophilic glucose degradation products from the glycosylated proteins and other small-molecular-weight products. Following this procedure, the allergenic activity of the protein itself, and not of other compounds formed during roasting, was assessed. Finally, the protein concentration was assessed using the BCA assay and aliquots were stored at -70°C . Samples will be referred to as N (native), R+g (heated at low moisture in the presence of glucose) or R-g (heated at low moisture in the absence of glucose).

Biochemical characterization

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot analysis. SDS-PAGE (12%) was performed under reducing conditions on a mini-protein II gel apparatus (Bio-Rad, Veenendaal, the Netherlands) [31]. The gels were stained using the Gel Code blue stain (Pierce, Rockford, IL, USA) and the precision plus protein dual colour MwM was used as the molecular weight standard (Bio-Rad).

For the analysis of IgE-binding fragments of the thermally processed Ara h 1 and Ara h 2/6, a Western blot analysis was performed using a plasma pool of the first nine peanut-allergic patients described in Table 1. SDS-PAGE (12%)-separated proteins were electrophoretically transferred to Protan nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were blocked in Tris-buffered saline with Tween-20 [TBST; 20 mM Tris, 500 mM NaCl, 0.05% (v/v) Tween, pH 7.5] plus 3% BSA for 1 h at room temperature and then incubated with a 1 : 15 dilution of the pooled human plasma overnight at 4°C in 0.5% BSA in TBST. After incubation for 1 h with biotin-labelled mouse anti-human IgE (1 : 250, BD Pharmingen, San Diego, CA, USA) in TBST plus 5% non-fat dry milk, membranes were washed and bound IgE was detected by streptavidin poly-horse radish peroxidase (1 : 4000, Sanquin, Amsterdam, the Netherlands) in TBST plus 5% milk powder for 1 h at room temperature. Between each incubation step, membranes were washed twice with TBST/Triton [TBST, 0.2% (v/v) Triton X-100]

Table 1. Serological and clinical information for the 12 peanut-allergic patients recruited from the Allergology Practice Arnhem, the Netherlands

Code	Age [years/sex (f/m)]	Grade peanut allergy	SPT (−/+ /++)	Other food allergies	Total IgE (kU/L)	Specific IgE (kU/L)		
						Peanut (f13)	Ara h 1	Ara h 2/6
65	19/f	3/4	++	No	787	> 100	267	208
66	18/m	4	++	No	514	> 100	173	71
70	21/f	3/4	++	Soy, pea, apple	1869	> 100	117	76
78	24/f	3	++	HN	406	79.6	62	29
67	46/f	3	ND	HN, kiwi, cherries	3986	54.4	51	35
73	22/f	3/4	++	HN	96	33.6	19	3.7
77	25/m	2	++	No	86	33.2	21	3.5
68	20/f	1	++	No	109	17.6	10	6
80	27/m	3	++	No	110	16	6.9	17
76	23/m	3	++	No	108	8.59	6	0.3
69	21/m	3/4	++	No	200	6.98	2.2	1.2
82	18/f	1/2	+	Kiwi, cherries	23	4.75	2.3	6.4

IgE concentrations (IU/mL) of the total IgE and IgE specific for Ara h 1 and Ara h 2/6 were determined by EAST assay on anti-IgE or allergen-coated plates. Each serum was tested as serial dilutions from 1/4 to 1/2500, in duplicate. The limits of detection of the assays were 0.1 IU/mL, allowing detection values > 100 kU/L. Specific IgE for peanut (f13) was determined using ImmunoCAP (Phadia AB) and values were capped at 100 kU/L.

Grading is based on Brockow and Ring [24] and on Sampson [25]. Grade 1, dermal symptoms; grade 2, gastro-intestinal problems such as nausea and/or cramping; grade 3, any of the former grades plus vomiting/diarrhoea and respiratory tract problems such as throat pruritis or tightness; grade 4, any of the former grades and respiratory arrest plus cardiovascular problems such as hypotension.

ND, not determined; HN, hazelnut.

and once with TBST, for 10 min at room temperature. Signal was detected by development using a chemiluminescence kit (Amersham Biosciences, Roosendaal, the Netherlands) according to the manufacturer's protocol and visualized using the Lumi-film chemiluminescent Detection Film (Roche, Mannheim, Germany).

Protein size measurement by light scattering. The measurements of the average size were carried out on a Malvern Zetasizer Nano-ZS analyser (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Dynamic light scattering (DLS) was performed and the intensity of the light scattered by the protein was measured at an angle of 173° .

Samples were measured in disposable micro cuvettes (Malvern Instruments Ltd.) at a concentration of $250\text{ }\mu\text{g/mL}$ in sterile PBS, pH 7.4. First, the sample was equilibrated for 300 s at 20°C , after which a time scale of the scattered light intensity fluctuations was measured. Every sample was measured in triplicate and autocorrelation analysis was carried out using the Zetasizer software for the Nano, v6.20 (Malvern Instruments Ltd.). Data were processed using multiple narrow modes (high resolution) as an analysis model, which yielded the lowest multimodal fit error. Data are presented both as size distribution by volume as by intensity.

***o*-Phthaldialdehyde assay.** The *o*-phthaldialdehyde (OPA) assay was applied to determine the quantity of free amino groups according to Nielsen *et al.* [32], with slight modifications. The protein solution ($250\text{ }\mu\text{g/mL}$) was mixed with the reagent in a ratio of 1 : 10 (v/v) and incubated for 20 min at room temperature. The absorbance was measured at 340 nm and samples were measured in triplicate. Unreacted amino groups were estimated from a calibration curve established with L-leucine.

Analysis of the immunoglobulin E immunoreactivity

The concentrations of total IgE and of IgE specific to purified Ara h 1 and Ara h 2/6 were determined using the enzyme allergosorbent assay (EAST) as described previously [21, 33].

Enzymatic Ara h 1 tracer was prepared by covalent linkage of N-Ara h 1 to the tetrameric form of acetylcholinesterase (AChE) using *N*-succinimidyl-*S*-acetylthioacetate (SATA) reacting with maleimido groups previously incorporated into AChE with *N*-succinimidyl-4-(maleidomethyl)-cyclohexane-1-carboxylate (SMCC) as described [34, 35]. N-Ara h 2/6 was dialysed against borate buffer 0.1 M pH 9 (NMWL 3500 Da), and then incubated for 45 min at room temperature with EZ-link sulpho-NHS-LC biotin (Pierce, Rockford, IL, USA) using a 10 : 1 molecular ratio biotin : N-Ara h 2/6. Biotinylation was stopped by further incubation for 1 h in 1 M Tris buffer, pH 8.5.

Biotinylated N-Ara h 2/6 were then dialysed and diluted in EIA buffer and maintained at 4°C until use.

Inhibitions were performed in a reverse EAST [34]. Briefly, $50\text{ }\mu\text{L}$ /well of different dilutions of each serum that were optimized in an initial experiment were distributed on plates coated with the anti-human IgE (mouse monoclonal, clone LE27; described by Grassi *et al.* [36]) and incubated overnight at 4°C . After washing, $25\text{ }\mu\text{L}$ of inhibitor and $25\text{ }\mu\text{L}$ of tracers were added and incubated for 4 h at room temperature. Inhibitors consisted of increasing concentrations of N-, R+g or R-g Ara h 1 (10^{-4} – $100\text{ }\mu\text{g/mL}$) or Ara h 2/6 (10^{-5} – $10\text{ }\mu\text{g/mL}$). Enzymatic tracers consisted of N-Ara h 1 labelled with AChE or biotinylated N-Ara h 2/6 (200 ng/mL). When using a biotinylated tracer, an additional 45-min incubation with $50\text{ }\mu\text{L}$ of streptavidin labelled with AChE was performed. After washing, Ellman's reagent was used as an enzyme substrate [37]. Results were expressed as B/B_0 , where B_0 and B represent the amount of N-Ara h 1 or N-Ara h 2/6 tracers linked to immobilized IgE in the absence or presence of a known concentration of inhibitor, respectively.

Indirect basophil histamine release

Buffy coats were obtained from the hospital blood bank in Copenhagen and cells were screened and selected for a high anti-IgE response and no allergies. Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep gradient centrifugation, cell-bound IgE was stripped from the cells by a phosphate buffer and basophil histamine release (BHR) tests were performed as described earlier [38]. Six dilutions (0.001 – $100\text{ }\mu\text{g/mL}$) were tested for Ara h 1 and 12 (0.0002 – $20\text{ }\mu\text{g/mL}$) for Ara h 2/6 and the concentration range chosen was based on earlier optimization experiments. Allergens were graded based on the lowest concentration inducing a histamine release (HR) of $>10\text{ ng/mL}$, in which grade 1 represents basophils only reacting to the highest concentration of the allergen tested and 6 represents basophil reaction to the lowest concentration tested.

Mediator release assay using rat basophilic leukaemia cells

Rat basophilic leukaemia (RBL)-2H3 cells expressing the α -chain of the human Fc ϵ RI receptor [39] were kindly provided by Drs Vieths and Vogel (Paul-Ehrlich-Institut, Langen, Germany). In a preliminary experiment, four sera dilutions (1 : 20, 1 : 30, 1 : 40 and 1 : 50) and three concentrations of a peanut extract (0.01, 0.1 and $1\text{ }\mu\text{g/mL}$) were tested for all 12 sera and suitable sera were selected based on the percentage β -hexosaminidase release. Cells were cultured in MEM medium supplemented with 5% fetal calf serum and 1% glutamine (all from Gibco, Paisley,

UK) at 37 °C in a humidified atmosphere with 5% CO₂. Cells in the stationary growth phase (confluent or slightly overgrown) were harvested and plated in 96-well plates at 1.5×10^5 cells/well. Human sera (1 : 30) were added and incubated overnight to sensitize the cells passively. After washing, the cells were stimulated for 1 h with the allergens diluted in Tyrode's buffer containing 50% deuterium oxide [39]. The antigen-specific release was quantified by measuring β -hexosaminidase activity and expressed as percentage of the total β -hexosaminidase content that was obtained by lysing the cells with 1% Triton X-100 (Sigma-Aldrich, Zwijndrecht, the Netherlands) [40]. Spontaneous release was determined on cells not sensitized or cross-linked. The toxicity of each serum was also checked using sensitized cells not further cross-linked with allergens. The release data were fitted to four-parameter-logistic curves by non-linear regression using the SigmaPlot v10.0 software package and IC₅₀ values were assessed accordingly.

Statistical analysis

Because of a non-normal distribution of the data, the non-parametric Wilcoxon's signed-rank test was used to compare the IC₅₀ values and allergen classes between the native and treated allergens. The difference was considered statistically significant for $P < 0.05$. The statistical analysis was performed using SPSS Software (v15.0, SPSS Inc., Chicago, IL, USA).

Results

Effect of model thermal treatment on allergen structure

Samples heated in the presence of glucose turned brown due to the formation of Maillard browning products. Soluble proteins were recovered from the thermally treated proteins and this yield differed for the individual proteins. Around 75% of the Ara h 1 and 53% of the Ara h 2/6 remained soluble after heating without glucose. The addition of glucose resulted in a dramatic loss of soluble protein for Ara h 1, with only 5% of the protein being recovered. Ara h 2/6 solubility (58%) was very similar to that of the sample heated without glucose (data not shown).

Analysis of the residual soluble Ara h 1 showed that heating had resulted in significant hydrolysis of the protein (Fig. 1a), with the appearance of two new polypeptides of about 23 and 33 kDa and substantial material of < 15 kDa appearing at the gel dye front after heating in the absence of glucose. This was accompanied by a loss of the parent 63.5 kDa polypeptide. The faint smeared appearance is indicative of some level of polypeptide aggregation and the formation of numerous heterogeneous structures due to different stages of the Maillard reaction.

The addition of glucose during the heating resulted in a loss of any polypeptides resolvable by SDS-PAGE with all the stained proteins having an $M_r < 15$ kDa and a faint smear present in the protein-stained gel, suggesting that heating in the presence of glucose had resulted in more extensive degradation. However, part of the protein did not enter the SDS-PAGE due to large aggregate formation, as substantiated by the light scattering results. The degradation products in both samples were still able to bind IgE antibodies from the peanut-allergic patients in the IgE-immunoblot experiment (Fig. 1c) despite R+g Ara h 1 being present at such low levels that they were barely discernable on the protein-stained gel (Fig. 1a). The thermal treatment also resulted in the degradation of Ara h 2/6 to low-molecular-weight polypeptides below the resolving capacity of the SDS-PAGE gel used, with a substantial amount of protein staining observed at the dye front of the gel (Fig. 1b). Heating in the presence of glucose resulted in the protein being poorly resolved, with a smear evident in the protein-stained gel. The pyrolysis products of the Ara h 2/6 were able to bind IgE, with individual polypeptides discernable on the Western blot (Fig. 1d), showing that Ara h 2/6 also retained its IgE-binding capacity even after such an extreme thermal treatment.

The content of free amino groups was significantly lower in the heated Ara h 1 samples and the lowest in the Ara h 1 heated in the presence of glucose as determined by the OPA assay (Table 2). For Ara h 2/6, roasting did not significantly change the free amino groups content significantly (data not shown).

The effect of thermal treatment on the aggregation state of the soluble fraction of the Ara h 1 was also assessed using fixed-angle dynamic light scattering (Table 2, Fig. 2). The unheated N-Ara h 1 sample had a mean hydrodynamic diameter of around 15 nm, consistent with the protein being present in its native trimeric state. Heating alone resulted in the soluble fraction of Ara h 1 having a slightly reduced size of around 12 nm, consistent with the hydrolysis of the protein observed by SDS-PAGE, although part of these degraded polypeptides were clearly assembled into much larger aggregated structures, ranging in size from 30 to 400 nm analysed by intensity (Fig 2b). The soluble fraction remaining after heating in the presence of glucose was present as an ensemble of very large aggregated structures ranging in size from ~200 to 1000 nm, with an average diameter of 560 nm (Table 2, Fig. 2). Because of the relatively small dimensions of Ara h 2/6, this type of analysis was not performed for the 2S albumins.

Allergenic activity of thermally modified peanut allergens

Nine of the 12 peanut-allergic patients (five males and seven females, median age: 22 years) showed grade 3 or 4

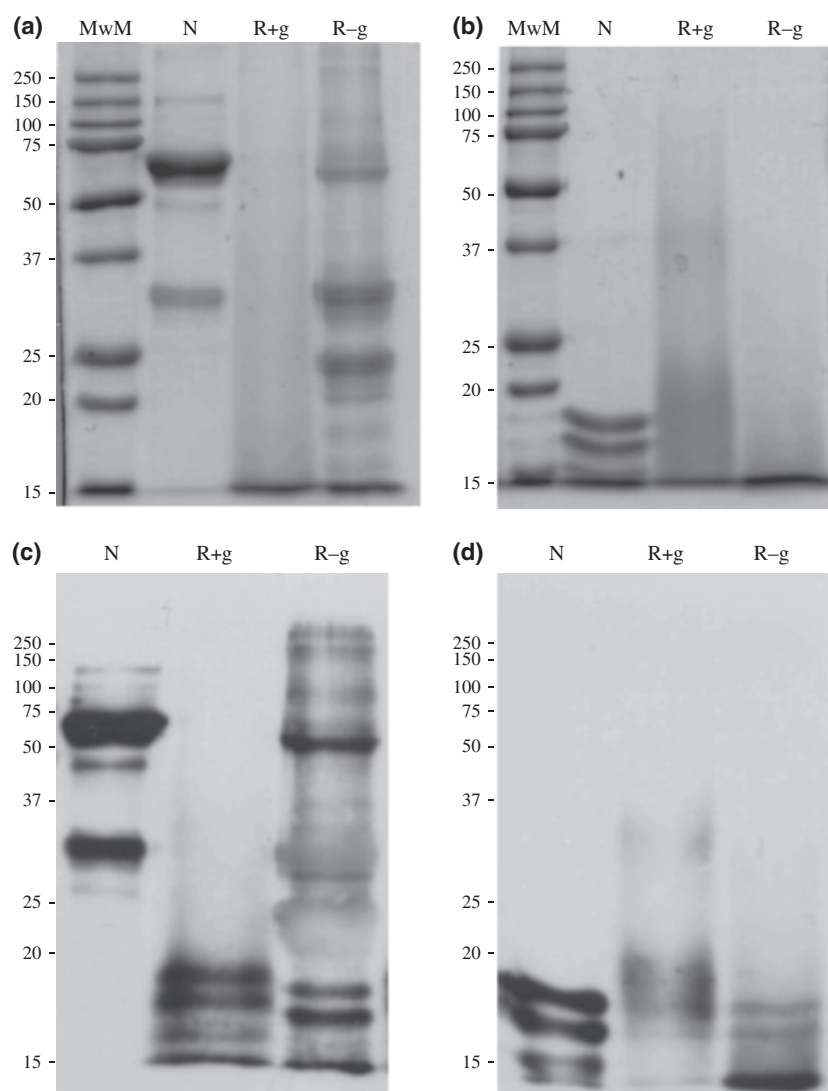


Fig. 1. SDS-PAGE and Western blot of the native and thermally processed Ara h 1 and Ara h 2/6 proteins. Ara h 1 (a) and Ara h 2/6 (b) were run on SDS-PAGE and stained with Coomassie blue stain. The same samples were transferred to nitrocellulose and analysed by Western blot analysis using a pool of plasma from nine peanut-allergic patients. The bound plasma IgE was recognized by biotin-labelled mouse anti-human IgE in combination with strep poly HRP and visualized by chemoluminescence (c and d). MwM, molecular weight standard; R-g, heated in the absence of glucose; R+g, heated in the presence of glucose.

Table 2. Biochemical analysis of native and heated allergens

	N Ara h 1	R+g Ara h 1	R-g Ara h 1
Percentage-free primary amino groups in relation to the native allergen (mean±SD)	100	16.1±1.4	35.1±3.6
Mean size±width [<i>d</i> (nm)]*	15.1±3.2	563±191	12.1±2.6
<i>Z</i> _{av} [<i>d</i> (nm)] [†]	16.2±0.1	389±13.3	45±0.5

*Analysed as size distribution by volume of the most common peak that represented for all samples >98% of the total volume.

[†]*Z*-average (mean±SEM) of triplicate measurements; analysed as size distribution by intensity and including all peaks.

clinical peanut allergy and all except one showed a highly positive (++) SPT test (Table 1). Specific IgE values for peanut extract, Ara h 1 and Ara h 2/6 were assessed in the sera of the patients (Table 1). All patients were

polysensitized for the different peanut allergens tested (Ara h 1 and Ara h 2/6), which is in accordance with a recent study that showed only a low percentage (7%) of mono-allergen-sensitized peanut-allergic patients [4].

IgE immunoreactivity of heated Ara h 1 and Ara h 2/6 was analysed by reverse EAST inhibition. Control experiments were performed using sera from non-allergic subjects and showed no IgE binding at all. Figure 3 shows inhibition of the IgE binding to N-Ara h 1 (Fig. 3a) and N-Ara h 2/6 (Fig. 3b) by N, R+g and R-g allergens obtained for representative sera. The IC_{50} values obtained for the different sera and allergens were then calculated (Fig. 4). Heating decreased the IgE-binding capacity of Ara h 1, the IC_{50} values for R+g and R-g Ara h 1 being ~9000- and

3.6-fold higher, respectively, compared with the N-Ara h 1. Heating also decreased the IgE-binding capacity of Ara h 2/6, resulting in a 22- and almost a 600-fold decrease of the inhibition potency for R+g and R-g Ara h 2/6, respectively, when compared with N-Ara h 2/6.

The IgE cross-linking capacities of native and heated allergens were initially determined using HR tests that were performed using passively sensitized human stripped basophils. Sera from the selected peanut-allergic patients were first screened to determine the optimal concentration range of the allergens. Sera from two non-allergic controls were included for which HR levels were < 10 ng/mL for all allergens (data not shown). HR dose-response curves were similar for all patients' sera tested and the

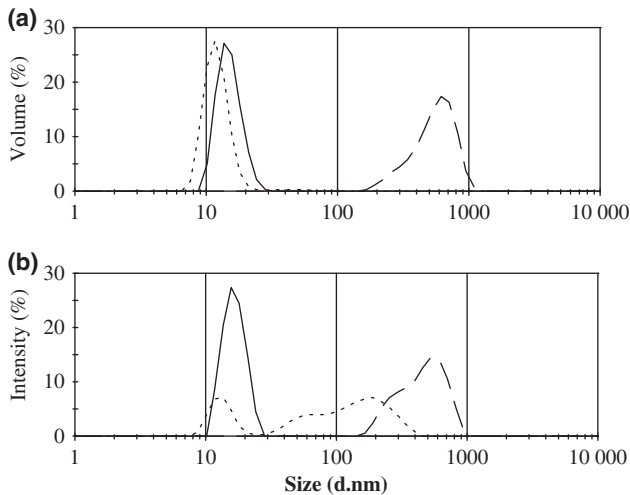


Fig. 2. Particle size distribution of N, R+g, and R-g Ara h 1 measured with dynamic light scattering. Light scattering measurements of native (N; —), heated in the presence of glucose (R+g; ----) or heated in the absence of glucose (R-g; ····) Ara h 1 protein particles. Results are shown as size distribution by (a) volume, percentage volume vs. size in nm and (b) intensity, percentage intensity vs. size in nm. Data represent the mean of three measurements per sample.

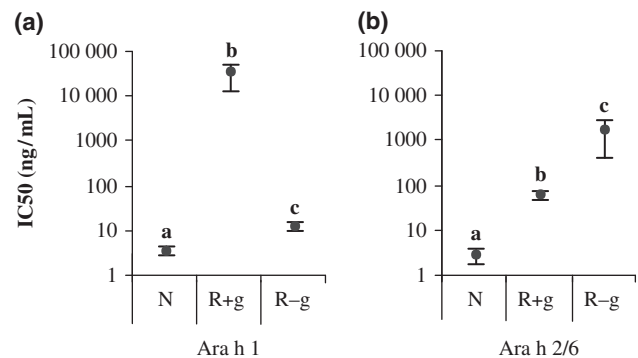


Fig. 4. Analysis of the IC_{50} (ng/mL) values obtained using an IgE capture inhibition assay with N, R+g, and R-g Ara h 1 (A) and N, R+g, and R-g Ara h 2/6 (B) as competitors. Protein amounts to obtain 50% inhibition of the Native Ara h 1 tracer (a) or the Native Ara h 2/6 tracer (b) were determined using five (a) or eight (b) individual sera from peanut-allergic patients. The average IC_{50} values are shown $\pm$ SEM. An increase of the IC_{50} value corresponds to a decrease in IgE-binding capacity. Means without a common letter differ ($P < 0.05$).

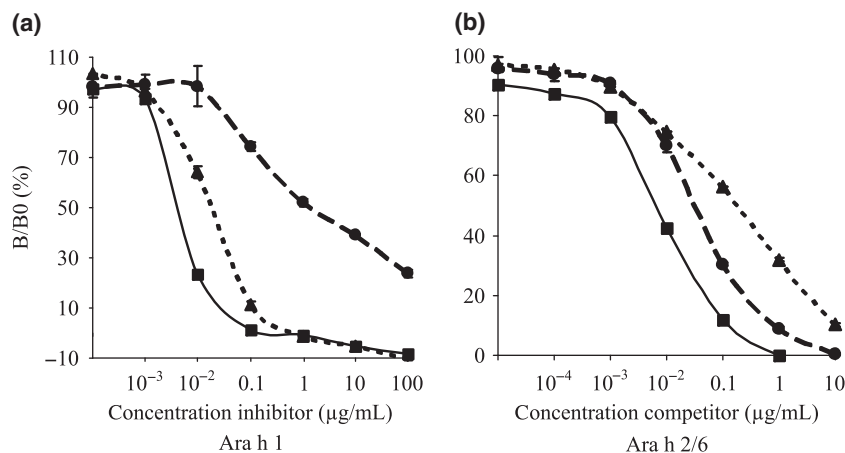


Fig. 3. IgE capture inhibition curves obtained for N, R+g, and R-g Ara h 1 (A) and N, R+g, and R-g Ara h 2/6 (B) as competitors. IgE-binding capacities of native and heat-modified $\pm$ glucose allergens were assessed by competitive assays in the IgE capture format. Inhibition was performed into microtitre plates coated with anti-IgE, and previously incubated with allergic patient sera at a convenient dilution. Competition was conducted accordingly, by adding increasing concentrations of competitors, i.e. native (N; —■—), heated in the presence of glucose (R+g; ---●---) or in the absence of glucose (R-g; ---▲---) allergens, at the same time as labelled native allergens [Ara h 1 (a) or Ara h 2/6 (b)]. Results are shown of representative sera of peanut-allergic patients (serum #78 for Ara h 1 and serum #70 for Ara h 2/6). Averages are shown of duplicate values $\pm$ SD.

titration curves of representative sera are represented in Figure 5. N-Ara h 2/6 (Fig 5b) demonstrated a profoundly higher biological activity than N-Ara h 1 (Fig 5a), showing an HR of >10 ng/mL at a protein concentration of 0.001 μ g/mL compared with the 10 μ g/mL of N-Ara h 1 needed to elicit equivalent HR. Despite reducing the IgE-binding capacity of Ara h 1, heating in the presence or absence of glucose profoundly increased the degranulation potency of this allergen, and showed an HR of >10 ng/mL at a concentration of 0.01 μ g/mL heated both in the presence and absence of glucose (Fig 5a). In contrast, heating reduced the degranulation activity of Ara h 2/6, particularly in the absence of glucose (Fig 5b). The different proteins were graded from 1 to 6, a higher grade indicating a higher histamine-releasing capacity. Thus, Ara h 1 showed a significantly higher allergen class after heating (independent of the addition of glucose), while Ara h 2/6 had a significantly lower allergen class after heating in the absence of glucose.

The biological mediator-releasing activity of the native vs. the heat-modified allergens was also assessed in the RBL MRA. The sera that showed significant degranulation capacity in this test were generally those sera having high peanut-specific IgE levels. After passive sensitization of RBL cells with IgE from peanut-allergic patients, the cells were challenged by serial dilutions of the native or processed allergens, which resulted in a dose-dependent mediator release. Figure 6 shows the typical results of representative sera. In addition, the table presented within each figure represents the average protein concentrations to induce 50% of the maximum mediator release induced by the native allergen (EC_{50}). The allergenic activity of the thermally treated Ara h 1 was dramatically increased, with a 700- and 1700- fold reduction in EC_{50} for R+g and R-g

Ara h 1, respectively, compared with the native protein (Fig 6a). The presence of glucose during heating had only a minor influence on the allergenic activity. Similar results were obtained with respect to light scattering, IgE reactivity and RBL degranulation capacity with a different batch of heated and heat-glycated Ara h 1 (data not shown).

In contrast, extensive heating of Ara h 2/6 increased the EC_{50} values by 19- and 661-fold for R+g and R-g, respectively (Fig 6b). The presence of glucose significantly moderated this loss in degranulation capacity over heating alone. Non-specific binding to the protein (and possibly other products present after Maillard modification) was not observed as no β -hexosaminidase release was induced by the different allergens when using sera from non-allergic patients (data not shown). Furthermore, testing the native allergens, N-Ara h 2/6 showed the highest degranulating activity, with a maximum release obtained at 1 ng/mL vs. 1 μ g/mL for N-Ara h 1.

Discussion

Scarce literature exists on the effect of processing methods on peanut proteins and published studies mostly use boiled, fried or roasted peanuts from which allergens are extracted and tested for their allergenicity [21, 41–44]. To study the effect of processing on specific proteins, this method has several drawbacks such as the frequent use of denaturing agents to extract the proteins from the processed peanuts, which might affect the structure and thus the allergenicity of the protein. In addition, insolubility even to denaturing solvents might result in extracting and testing only a specific, soluble fraction of the proteins and differences in aggregation state and resulting protein

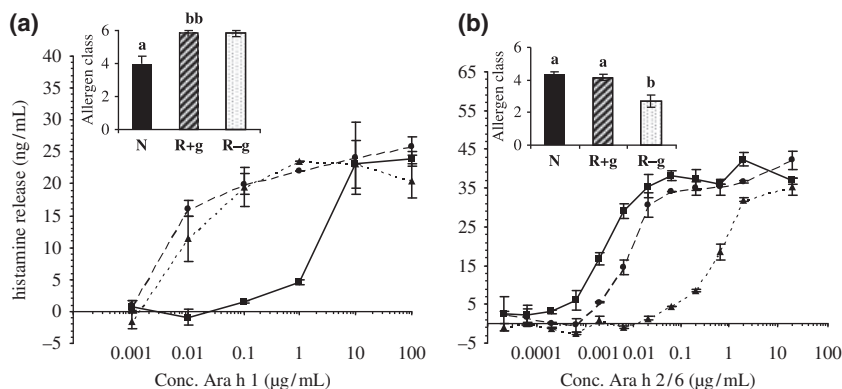


Fig. 5. Histamine release (ng/mL) by passively sensitized basophils induced by (non-) modified allergens. Basophils were passively sensitized with sera from a peanut-allergic patient and stimulated with increasing concentrations of native (N; —■—), heated in the presence of glucose (R+g; ---●---) or in the absence of glucose (R-g; ---▲---) Ara h 1 (a) or Ara h 2/6 (b). Results are shown of representative sera of peanut-allergic patients (serum #67 for Ara h 1 and serum #69 for Ara h 2/6). Error bars represent the SD of duplicate values. The column graphs presented within each figure represent the classes of the allergens, based on grading of the responses from 1 to 6. The grading is based on the lowest concentration inducing a histamine release of >10 ng/mL, in which grade 1 represents basophils only reacting to the highest concentration of the allergen tested and 6 represents a basophil reaction to the lowest concentration tested ($n = 6-7$). Means without a common letter differ ($P < 0.05$).

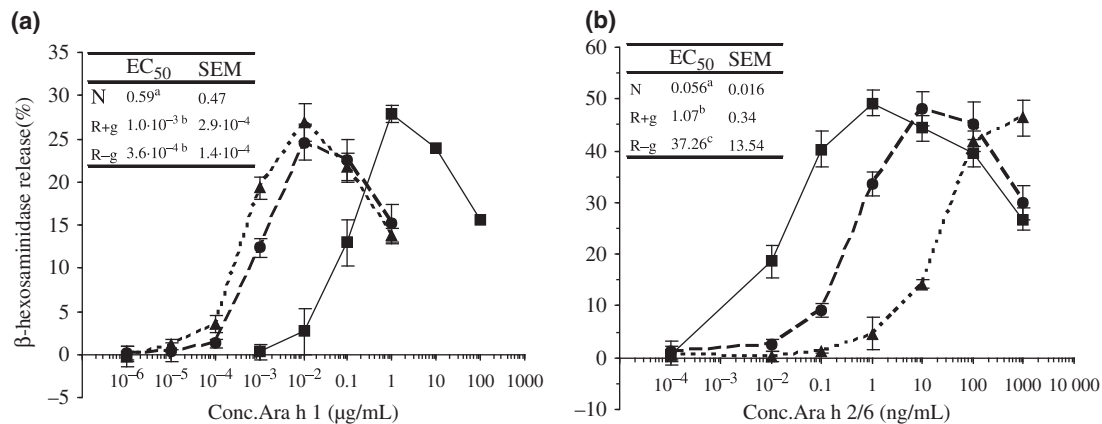


Fig. 6. Percentage of β -hexosaminidase release from humanized rat basophilic leukaemia (RBL) cells induced by (non) modified allergens. Humanized RBL cells were passively sensitized with sera from a peanut-allergic patient and stimulated with increasing concentrations of native (N; —■—), heated in the presence of glucose (R+g; —●—) or in the absence of glucose (R-g; —▲—) Ara h 1 (a) or Ara h 2/6 (b). Results are shown of representative sera of peanut-allergic patients (serum # 70 for Ara h 1 and serum # 66 for Ara h 2/6). Error bars represent the SD of triplicate values. The table presented within each figure represents the average protein concentrations (μ g/mL for Ara h 1 and ng/mL for Ara h 2/6) to obtain 50% of the maximum allergen release of the native allergen (EC₅₀) ($n = 4-5$). Means without a common letter differ ($P < 0.05$).

solubility will affect the protein composition of the extracted protein fraction [45]. After boiling, it was shown that low-molecular-weight proteins were transferred into the cooking water, which mainly caused the decrease in allergenicity observed in boiled peanuts [21]. In order to relate processing effects to the impact on structure of allergens more directly, we studied the effects of thermal treatments on the allergenic activity of Ara h 1 and Ara h 2/6 purified from raw peanut. Many thermal treatments used in the processing of peanuts, such as roasting and frying, are undertaken when the water content is low, usually around 4%. The present paper describes a model treatment for purified allergens that attempts to mimic these processing conditions, using freeze-dried allergens heated at 145 °C for 20 min in the presence or absence of glucose. The presence of water can have a profound effect on the denaturation behaviour of proteins as these tend to be more thermostable when the water content is limiting [46]. In the present study, the effect of the heating was on the one hand pyrolysis of the proteins, resulting in their degradation to much smaller polypeptides. On the other hand, mainly for Ara h 1 after extensive heating with glucose, aggregation of monomers resulting in large macromolecular aggregates not dissolvable in SDS might also have occurred. The greater thermal stability of the 2S albumins resulted in a greater proportion of the Ara h 2/6 being soluble after the application of heat in the dry state compared with Ara h 1, irrespective of whether glucose was included. In addition, covalent cross-linking of the protein as a consequence of Maillard reactions [17, 47] explains the low protein extraction yield of Ara h 1 heated in the presence of glucose. The soluble fractions used in the current study represent parts of the proteins that might be readily soluble from roasted peanuts and hence involved in the rapid reactions observed in peanut-allergic

individuals, particularly in the oral cavity. These were observed to be present in an aggregated form but comprised thermally induced hydrolysis products, as almost no intact Ara h 1 or Ara h 2/6 remained after the thermal treatment.

The decrease in the IgE-binding capacity of the thermally treated peanut allergens indicates structural changes induced by heating that results in epitopes having either reduced affinity and/or accessibility for specific IgE. This was particularly marked for Ara h 1 heated in the presence of glucose, which, on the basis of the loss of primary amino groups, suggests that non-enzymatic glycosylation of the protein changed or destroyed the epitopes. Alternatively, shielding of the epitopes by covalently attached polysaccharide chains or by the higher extent of aggregation may render it less able to bind IgE. In addition, the hydrophilic glucose could have altered the hydrophobicity of the protein, resulting in a decrease in IgE binding as the hydrophobic amino acid residues located in the centre of the epitope appeared to be the most critical for IgE binding [10].

Besides determining the IgE-binding capacity of the allergen, we also assessed the biological activity of the native and processed allergens, i.e. their potency to cross-link cell-bound IgE and to induce effector cell degranulation. Even though a highly significant decrease in IgE-binding capacity was observed for heated Ara h 1, its capacity to elicit mediator release was significantly enhanced. Theoretically, more than one IgE-binding site is required per fragment of an allergen to induce IgE cross-linking. The R+g Ara h 1 aggregates present a large surface containing multiple copies (of the same) IgE epitopes, which clearly enhances the cross-linking capacity of the protein, in comparison with its intrinsic IgE-binding capacity, as defined by immunoassays. There is

other evidence showing that aggregated structures are more effective in triggering HR than soluble monomeric forms of the same protein [48]. As both R+g Ara h 1 and R–g Ara h 1 had a strongly increased degranulation capacity, we can conclude that the Maillard reaction is of less importance for its cross-linking capacity and that these aggregated Ara h 1 structures, having a hydrodynamic diameter of at least 30 nm, are better in degranulating cells than Ara h 1, its native trimeric state having a diameter of 16 nm.

In contrast to Ara h 1, Ara h 2/6 does not form large aggregates, but does form dimers and tetramers following heat-induced denaturation [27, 45]. Although most studies that investigated the effect of processing on allergenicity assessed only the IgE-binding capacity, Lehmann *et al.* [49] also studied IgE cross-linking capacity. They observed that even though the IgE antibody-binding capacity of peanut 2S albumins was decreased after protease treatment, this did not result in a reduction of the allergenic potential using RBL cells. In our study, heating at 145 °C for 20 min decreased IgE binding as well as the IgE cross-linking capacity of Ara h 2/6, and the presence of glucose during roasting moderated these losses. This protective effect of glucose was also observed both for the IgE binding and the biological activity for the non-specific lipid transfer protein from apple (Mal d 3) after heating at 100 °C for 2 h [23]. Furthermore, we showed that heating had a different effect on Ara h 1 compared with Ara h 2/6 with respect to cross-linking capacity. Heating of Ara h 1 resulted in an increased cross-linking and degranulation capacity, while this was decreased after heating for Ara h 2/6. These data demonstrate that only studying the effect of food processing using IgE-binding capacity tests is insufficient to fully assess the degree of allergenicity of the (modified) protein and that effects on the biological activity, such as mediator release, must also be considered.

In summary, the effect of roasting on allergenicity in the presence or absence of glucose depends on the

allergenic protein tested and cannot be generalized for all proteins. We observed that in both cellular MRAs used in this study, heating at low moisture of purified Ara h 1 in the presence or absence of glucose resulted in an increased degranulation capacity, while for Ara h 2/6, the degranulation capacity was decreased. In addition, in contrast to Ara h 2/6, the IgE-binding capacity of Ara h 1 did not correlate to IgE cross-linking capacity and conclusions drawn from individual assays have to be considered with caution. Gaining more knowledge on the effect of the food matrix and the role of food processing on individual allergenic proteins will enable better understanding of their clinical impact, e.g. in terms of severity of reactions. It is also of importance in diagnostic procedures, in particular when food challenges are part of the diagnostic process. Threshold studies of peanut exposure may require standardization of the peanut roasting conditions to make the challenges used in clinical trials comparable. In addition, the results from this study can have important ramifications for approaches using purified components for component-resolved diagnosis.

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