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## RESEARCH ARTICLE

# Boiling peanut Ara h 1 results in the formation of aggregates with reduced allergenicity

Fany Blanc<sup>1\*</sup>, Yvonne M. Vissers<sup>2,3\*</sup>, Karine Adel-Patient<sup>1</sup>, Neil M. Rigby<sup>4</sup>, Alan R. Mackie<sup>4</sup>, A. Patrick Gunning<sup>4</sup>, Nikolaus K. Wellner<sup>4</sup>, Per S. Skov<sup>5</sup>, Laetitia Przybylski-Nicaise<sup>1</sup>, Barbara Ballmer-Weber<sup>6</sup>, Laurian Zuidmeer-Jongejan<sup>7</sup>, Zsolt Szépfalusi<sup>8</sup>, Janneke Ruinemans-Koerts<sup>9</sup>, Ad P. H. Jansen<sup>10</sup>, Hervé Bernard<sup>1</sup>, Jean-Michel Wal<sup>1</sup>, Huub F. J. Savelkoul<sup>2</sup>, Harry J. Wichers<sup>2,3,11</sup> and E. N. Clare Mills<sup>4</sup>

<sup>1</sup>INRA, UR496, Unité d'Immuno-Allergie Alimentaire, Jouy-en-Josas, France

<sup>2</sup>Cell Biology and Immunology Group, Wageningen University and Research Centre, Wageningen, The Netherlands

<sup>3</sup>Food Chemistry Group, Wageningen University and Research Centre, Wageningen, The Netherlands

<sup>4</sup>Institute of Food Research, Norwich Research Park, Colney, Norwich, UK

<sup>5</sup>RefLab ApS, Copenhagen, Denmark

<sup>6</sup>Allergy Unit, Department of Dermatology, University Hospital Zurich, Zurich, Switzerland

<sup>7</sup>Department of Experimental Immunology, Laboratory of Allergy Research, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands

<sup>8</sup>Department of Pediatrics, Medical University of Vienna, Vienna, Austria

<sup>9</sup>Department of Clinical Chemistry and Haematology, Rijnstate Hospital, Arnhem, The Netherlands

<sup>10</sup>Department of Otorhinolaryngology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

<sup>11</sup>Food and Biobased Research, Wageningen University and Research Centre, Wageningen, The Netherlands

**Scope:** Roasting rather than boiling and Maillard modifications may modulate peanut allergenicity. We investigated how these factors affect the allergenic properties of a major peanut allergen, Ara h 1.

**Methods and results:** Ara h 1 was purified from either raw (N-Ara h 1) or roasted (R-Ara h 1) peanuts. Boiling (100°C 15 min; H-Ara h 1) resulted in a partial loss of Ara h 1 secondary structure and formation of rod-like branched aggregates with reduced IgE-binding capacity and impaired ability to induce mediator release. Glycated Ara h 1 (G-Ara h 1) formed by boiling in the presence of glucose behaved similarly. However, H- and G-Ara h 1 retained the T-cell reactivity of N-Ara h 1. R-Ara h 1 was denatured, comprised compact, globular aggregates, and showed no evidence of glycation but retained the IgE-binding capacity of the native protein.

**Conclusion:** Ara h 1 aggregates formed by boiling were morphologically distinct from those formed by roasting and had lower allergenic activity. Glycation had no additional effect on Ara h 1 allergenicity compared with heating alone. Taken together with published data on the loss of Ara h 2/6 from boiled peanuts, this supports the hypothesis that boiling reduces the allergenicity of peanuts.

**Keywords:**

Allergenicity / Ara h 1 / EuroPrevall / IgE / Peanut allergy / Thermal processing

**Correspondence:** Professor E. N. Clare Mills, School of Translational Medicine, Manchester Academic Health Science Centre, Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, Manchester M1 7DN, UK

**E-mail:** clare.mills@manchester.ac.uk

**Fax:** +44-161-3065201

**Abbreviations:** AFM, atomic force microscopy; EAST, enzyme allergosorbent test; EC<sub>50</sub>, protein concentration needed to obtain 50% of the maximum mediator release induced by the native allergen; IC<sub>50</sub>, concentration of allergen able to inhibit antibody binding or histamine release by 50%; FT-IR, Fourier transform infrared spectroscopy; MRA, mediator release assay; NA, non-allergic; N-Ara h 1, native Ara h 1; PA, peanut-allergic; PBMC, peripheral blood mononuclear cells; RBL, rat basophilic leukemia

\*These authors contributed equally to this work.



## 1 Introduction

Peanut allergy is a common, severe and generally persistent food allergy in the North America and several European countries [1]. It appears to be less prevalent in other parts of the world, and it has been proposed that differences in cooking practices may be responsible [2]. Different types of thermal processing (blanching, boiling, roasting or frying) will alter protein structure in different ways, potentially changing its immunoreactivity and allergenicity. Since foods are complex mixtures of many different constituents, notably sugars, cooking can also modify proteins chemically. One of the most important of these cooking-related changes is the Maillard reaction, or non-enzymatic browning, which leads to the formation of stable advanced glycation end products (AGE) through the reaction of reducing sugars with free amino groups on proteins. It has been proposed that this type of processing can also affect the allergenicity of peanuts [2].

This has been particularly well studied for the peanut allergen Ara h 1, to which 55–95% of peanut-allergic (PA) patients are sensitized. Belonging to the 7S globulin protein family, it possesses the  $\beta$ -barrel structural motif characteristic of the cupin superfamily [3–6]. When purified from raw peanut it is a *M*, 210 kDa trimeric protein [7], composed of 63 kDa *N*-glycosylated subunits which can form multimers of up to *M*, 600–700 kDa depending on extraction conditions [8]. Like other cupins Ara h 1 is thermostable, only undergoing irreversible denaturation and extensive aggregation after passing through the main endotherm transition occurring above 80°C [9]. However, more extreme thermal processing such as roasting at 140°C appears to enhance IgE-binding capacity of Ara h 1 [10]. In model systems, heating native Ara h 1 for several days at 55°C in the presence of different sugars increased its IgE-binding capacity, which was related to the formation of AGE products although the IgE-binding capacity of the modified Ara h 1 and untreated allergen was not directly compared [11]. Such modifications may explain the enhanced IgE-binding capacity of Ara h 1 from roasted peanuts.

The problems of handling and investigating the structures of thermally aggregated proteins with reduced solubility has made it difficult to link the effects of thermal processing on structure to the effects on allergenic properties. In addition, few data are available comparing the functionality of modified and unmodified allergens. We have addressed this gap using structurally well-characterized thermally modified Ara h 1 preparations (including model processed Ara h 1 and the cognate protein from roasted peanuts). We investigated the effect of thermal treatment on both the allergen structure and its IgE-binding capacity, potency to stimulate histamine release and ability to induce T-cell proliferation and cytokine production using sera and peripheral blood mononuclear cells (PBMCs) from peanut allergic patients.

## 2 Materials and methods

### 2.1 Ethics statement

A written informed consent was obtained before the sample collection (serum or serum and PBMC), and the performed experiments were approved by the corresponding local ethical committees (Kantonale Ethikkommission Zürich, Medical ethical committee of the Amsterdam Medical Centre, Ethics Committee of Medical University of Vienna, Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen).

### 2.2 Patient sera and PBMC

Sera were obtained from a population of 35 PA patients (18 males and 17 females, mean age: 23 years) recruited from clinical centers in Zurich, Amsterdam, Arnhem and Vienna, or were provided by the EuroPrevall Serum Bank (EPSB; Supporting Information Table S1). Twelve PA subjects (underlined in Supporting Information Table S1) and 12 non-allergic (NA) controls, all recruited from the Allergology Practice Arnhem (APA, The Netherlands), donated cells for PBMC cultures. Peanut allergy was established using clinical history, physical examination, peanut-specific IgE (ImmunoCAP and/or RAST (Phadia AB; Supporting Information Table S1)) and objective clinical manifestations observed after peanut consumption (anaphylaxis in the history or positive food challenge).

### 2.3 Protein preparations

Whole peanut protein extract (WPPE) was prepared from peeled raw and commercially roasted peanuts (Virginian variety) from which R-Ara h 1 was purified [10]. Native Ara h 1 (N-Ara h 1) was purified from a single batch of peeled redskin-type raw peanuts (obtained from local suppliers, UK) as described by Marsh et al. [12]. N-Ara h 1 (4 mg/mL) in 32.5 mM phosphate buffer containing 100 mM NaCl was heated to 100°C alone (heated Ara h 1, H-Ara h 1) or in the presence of 100 mM glucose (glycated Ara h 1, G-Ara h 1) for 15 min. The protein concentration was verified by UV absorbance at 280 nm and an experimentally determined extinction coefficient of 0.572 absorbance units at 1.0 mg/mL at 280 nm in PBS. Protein solutions were allowed to cool to room temperature prior to analysis. The effect of thermal treatments was followed by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) analysis under reducing conditions with 50 mM 1,4-dithioerythritol using 4–12% gradient gels in a NuPAGE system (Invitrogen, Renfrewshire, UK) run for 35 min at 200 V in 2-(*N*-morpholino)ethanesulfonic acid buffer according to the manufacturer's instruction and loading 13  $\mu$ g protein per

lane (Supporting Information S1.1). Changes in secondary structure were defined using circular dichroism and Fourier transform infrared spectroscopy (Supporting Information S1.2 and S1.3; [12]). The content of a Maillard product (fructosamine) was determined using an assay based on nitroblue tetrazolium (Supporting Information S1.4). Aggregation state was defined using atomic force microscopy (AFM), depositing protein (40 µg/mL diluted 50-fold from a 2 mg/mL stock solution with water) on to freshly prepared poly-L-lysine coated muscovite mica (Agar Scientific, UK). The sample was inserted into the liquid cell of the AFM (East Coast Scientific, Cambridge, England) and imaged in a contact mode at a set point force of around 200 pN under redistilled *n*-butanol (Sigma, Dorset, UK) for the aggregates and buffer for the unheated proteins using 100 µm long oxide-sharpened Nanoprobe levers (NP-S, Veeco Instruments, CA, USA) with a quoted force constant of  $k = 0.38$  N/m. Complimentary analysis was undertaken using fixed angle light scattering carried out on a Zetasizer Nano (Malvern Instruments, Worcestershire, UK).

#### 2.4 IgE immunoreactivity of the native and modified Ara h 1 by direct and reverse EAST inhibition

IgE specific to whole protein extract from roasted or raw peanut and to N-Ara h 1 or R-Ara h 1 was determined using the enzyme allergosorbent test (EAST) as previously described on allergen-coated plates [3, 10]. Reverse EAST inhibition was performed as described elsewhere [13] using anti-human IgE (mouse monoclonal, clone LE27) coated plates. Inhibitors consisted of increasing concentrations of R-, N-, H- or G-Ara h 1 using either N-Ara h 1 or R-Ara h 1 labeled with acetylcholine esterase (AChE) [14] as the tracer. Results were expressed as  $B/B_0$ , where  $B_0$  and  $B$  represent the amount of Ara h 1 tracer linked to immobilized IgE in the absence or presence of a known concentration of inhibitor, respectively.

#### 2.5 Mediator release assay (MRA) using rat basophilic leukemia (RBL) cells

RBL-2H3 cells expressing the  $\alpha$ -chain of the human Fc $\epsilon$ RI receptor [15] were kindly provided by the Paul Ehrlich-Institut (Langen, Germany) and MRA were performed as described previously ([12], Supporting Information S1.5) using human sera annotated with an asterisk in Supporting Information Table S1. Antigen-specific release induced by N-, H- and G-Ara h 1 was quantified by measuring  $\beta$ -hexosaminidase activity and expressed as percentage of the total  $\beta$ -hexosaminidase content that was obtained by lysing the cells with 1% Triton-X100 (Sigma-Aldrich, Zwijndrecht, The Netherlands).

#### 2.6 The effect of thermal processing on PBMC-induced proliferation and cytokine production capacity

PBMCs were isolated from PA ( $n = 12$ ) and NA ( $n = 12$ ) donors and cultured according to previously described procedures [16]. No differences in viability, proliferation or cytokine secretion could be evidenced between PA and NA after polyclonal activation with  $\alpha$ CD3/ $\alpha$ CD28 (Supporting Information S2). PBMCs were stimulated with 20 µg/mL of N-, H- and G-Ara h 1 or with medium alone, cultured for 7 days at 37°C and supernatants were stored at -80°C (Supporting Information S1.6). Harvested PBMCs were characterized using flow cytometry using a FACSCanto II (BD Biosciences, San Diego, CA, USA) using labeled anti-CD4, anti-CD25 and anti-Ki-67 antibodies or the corresponding isotype controls, all from BD Pharmingen (San Diego, USA; Supporting Information S1.6). Cytokine production was determined using Cytometric Bead Array (CBA, BD Biosciences), according to manufacturer's protocol. The detection limits were 1.1 pg/mL for IL-5, 0.13 pg/mL for IL-10, 0.6 pg/mL for IL-13, 1.8 pg/mL for IFN- $\gamma$  and 0.3 pg/mL for IL-17.

#### 2.7 Statistical analysis

Means and medians were analyzed using non-parametric methods (Wilcoxon signed rank test or Mann-Whitney *U*-test) using SPSS Software (version 18.0, SPSS, Chicago, USA) or GraphPad Prism (version 4.00, GraphPad Software, San Diego, CA, USA) with a difference interpreted as significant when  $p < 0.05$ . MRA data were fitted to four-parameter-logistic curves by nonlinear regression using the SigmaPlot 10.0 and EC50 values determined.

### 3 Results

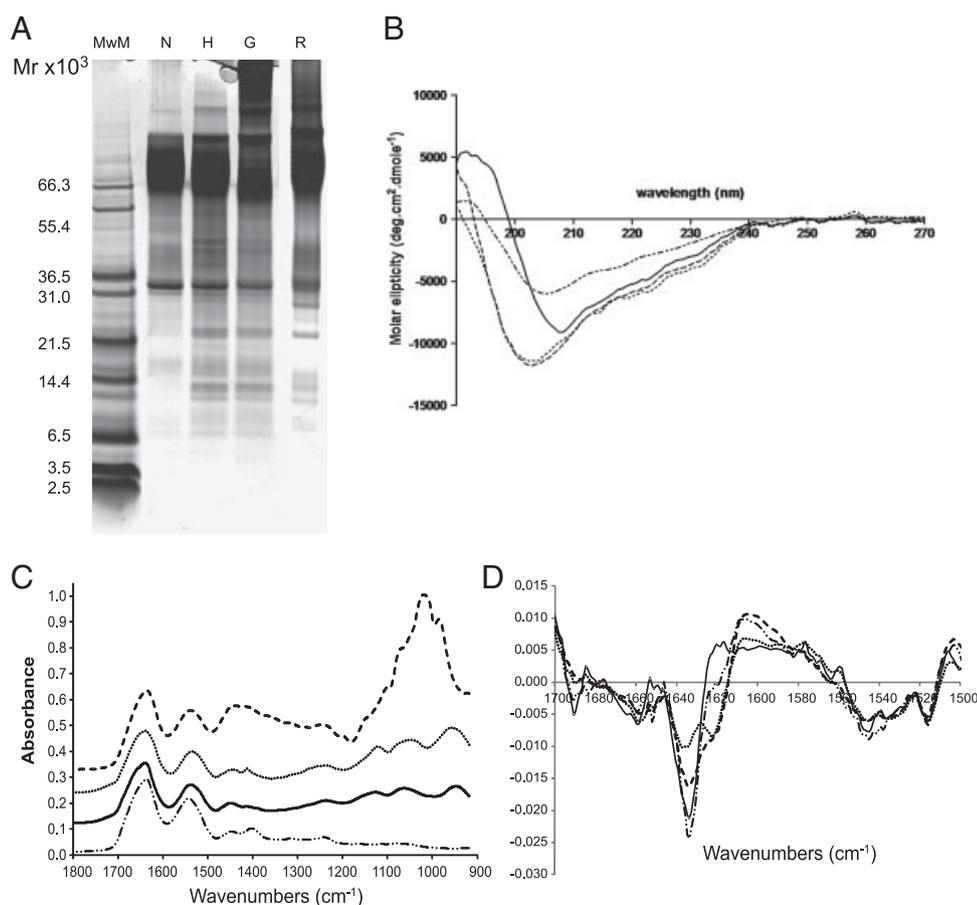
Boiling (H-Ara h 1) resulted in aggregation and hydrolysis of Ara h 1 as indicated by the appearance of lower  $M_r$  polypeptides ranging from ~6 to 67 kDa and the generally smeared appearance of the gel track (Fig. 1A, track 3) compared with the  $M_r$  ~67 and ~33 kDa polypeptides of native (N-) Ara h 1 (Fig. 1A, track 2) [12]. Addition of glucose during boiling (G-Ara h 1) resulted in the formation of high  $M_r$  polypeptides of masses > 200 kDa (Fig. 1A, track 4). Ara h 1 isolated from roasted peanuts (R-Ara h 1, Fig. 1A, track 5) resembled this heated and glycosylated protein, with much of the protein migrating as a poorly resolved smear at the top of the gel. Boiling, alone or in the presence of glucose caused a partial loss of secondary structure, as indicated by the loss of the positive maximum at 192–195 nm, and the positive molar ellipticity at 190 nm in the circular dichroism (CD) spectra of H- and G-Ara h 1 (Fig. 1B). However, the protein from roasted peanuts (R-Ara

h 1) appeared to be highly denatured having only a slight positive maximum at 190 nm and a negative minimum at 205 nm of reduced negative intensity (Fig. 1B). Analysis of the secondary structure content indicated that the protein had a much reduced  $\alpha$ -helical content of only 5.9% (compared with 22.3% for the native protein) and an increase in  $\beta$ -sheet content from 24.6 to 38.1%, the content of  $\beta$ -turn and unassigned structures remaining unaltered.

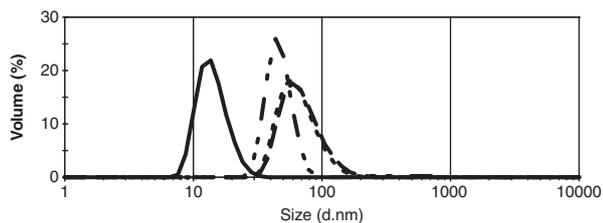
Complementary Fourier transform infrared spectroscopic analysis (FT-IR) was undertaken to provide additional information on secondary structure content and to obtain an indication of glycation state of the different protein preparations (Fig. 1C and D). The amide I band shape of N-Ara h 1 gave an amide I band maximum at  $1634\text{ cm}^{-1}$  characteristic of the natively folded  $\beta$ -sheet-rich protein, with a small shoulder at  $1656\text{ cm}^{-1}$  corresponding to  $\alpha$ -helix. After heating, the  $1634\text{ cm}^{-1}$  band was reduced in intensity and a new band appeared at  $1621\text{ cm}^{-1}$  which is generally accepted as an indication of the formation of intermolecular  $\beta$  sheet structures and protein aggregation [17]. Heating in the presence of glucose (G-Ara h 1) resulted in similar, but smaller, changes in secondary structure as well as glycation as indicated by the intense bands in the  $1200\text{--}900\text{ cm}^{-1}$  region. Roasting, i.e. heating at high temperature in low moisture environment, had a completely different effect.

The amide I band shape of R-Ara h 1 gave an even more pronounced amide I band maximum at  $1634\text{ cm}^{-1}$  characteristic of the natively folded  $\beta$ -sheet-rich protein, while the shoulder at  $1656\text{ cm}^{-1}$  corresponding to  $\alpha$ -helix was reduced. Only a small new band related to intermolecular  $\beta$ -sheet formation appeared at  $1621\text{ cm}^{-1}$ . Furthermore, the R-Ara h 1 had only a very minor absorption in the  $1200\text{--}900\text{ cm}^{-1}$  region, indicating a very limited level of glycation of the protein. These observations were confirmed by the determination of fructosamine content which showed N-Ara h 1 and R-Ara h 1 to contain  $\sim 10\text{ }\mu\text{M}$  fructosamine, whereas the G-Ara h 1 contained  $\sim 40\text{ }\mu\text{M}$  fructosamine. The concentration of fructosamine was low in these samples compared with the levels observed in samples heated in the absence of water at higher temperatures such as  $140^\circ\text{C}$ .

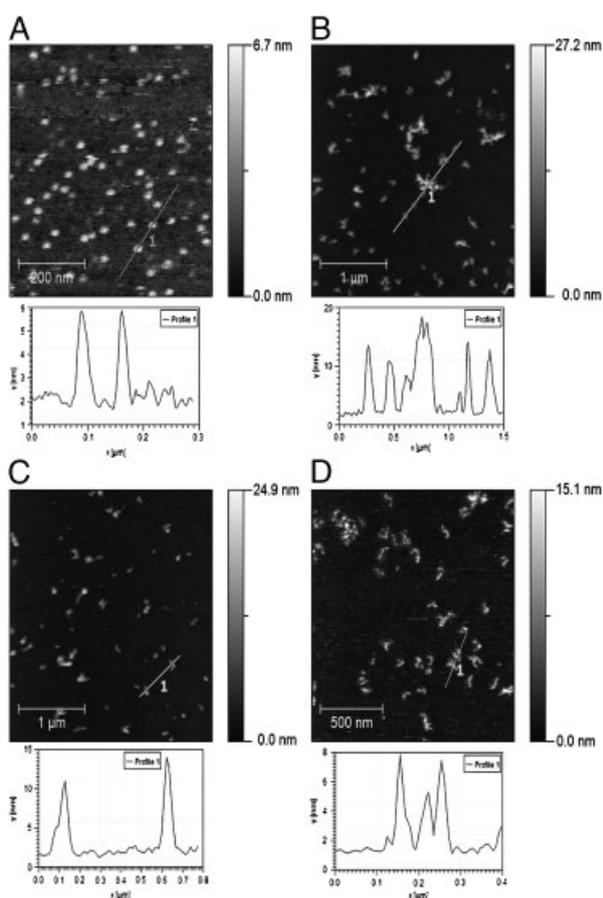
H-, G- and R-Ara h 1 were all highly aggregated with  $M_r$  of  $> 640\,000$  by gel permeation chromatography and dimensions of in excess of  $50\text{ nm}$  by fixed angle dynamic light scattering (Fig. 2). Characterization of boiled Ara h 1 aggregate topography using AFM showed that they had either a rod-like branched aggregate structure (H-Ara h 1, Fig. 3B) or simpler rod-like structures when glucose was added (G-Ara h 1, Fig. 3C) both with variable heights between  $10$  and  $15\text{ nm}$ . The R-Ara h 1 aggregates had a very different morphology (Fig. 3D) and were either more glob-



**Figure 1.** Physicochemical characterization of heat-treated Ara h 1. (A) SDS-PAGE analysis of Ara h 1 preparations. Track 1: Molecular weight markers; 2: native (N-) Ara h 1; 3: heated (H-) Ara h 1; 4: heated and glycosylated (G-) Ara h 1; 5: R-Ara h 1 from roasted peanuts. (B) Circular dichroism (CD) spectra of native and heat-treated Ara h 1. N-Ara h 1 (—); H-Ara h 1 (----); G-Ara h 1 (---); R-Ara h 1 (-----). (C) Fourier transform infrared spectroscopy (FT-IR) spectra of native and heat-treated Ara h 1. N-Ara h 1 (—); H-Ara h 1 (----); G-Ara h 1 (---); R-Ara h 1 (-----); (D) second derivatives of the amide I region of the Fourier transform infrared spectroscopy (FT-IR) spectra of native and heat-treated Ara h 1. N-Ara h 1 (—); H-Ara h 1 (----); G-Ara h 1 (---); R-Ara h 1 (-----).



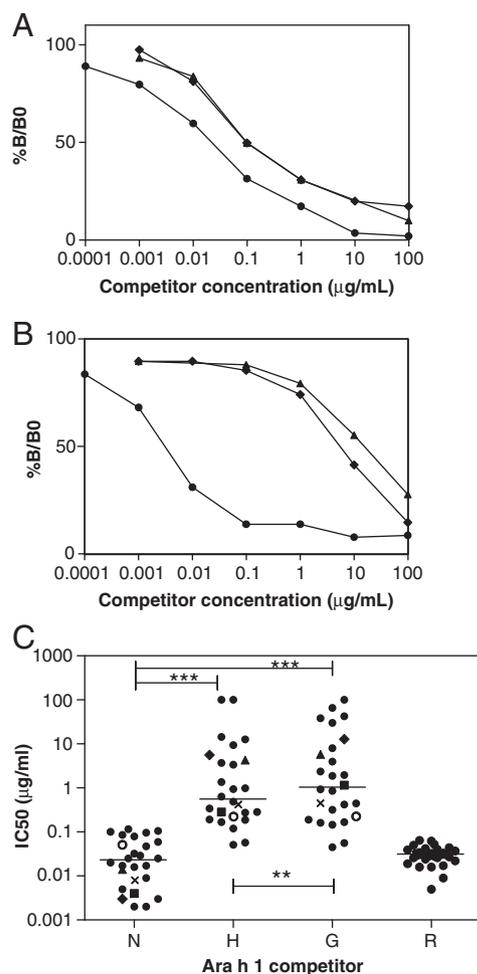
**Figure 2.** Fixed angle light scattering analysis of heat-treated Ara h 1. Light scattering measurements of native and heat-treated Ara h 1. N-Ara h 1 (—), H-Ara h 1 (----), G-Ara h 1 (- - -) or R-Ara h 1 (· · · ·). Results are shown as size distribution by volume, percentage volume versus size in nm. Data represent the mean of three measurements per sample.



**Figure 3.** Atomic force microscopy topographic images of heat-treated Ara h 1. (A) N-Ara h 1; (B) H-Ara h 1; (C) G-Ara h 1; (D) R-Ara h 1. Profiles beneath each image depict feature heights along marker line.

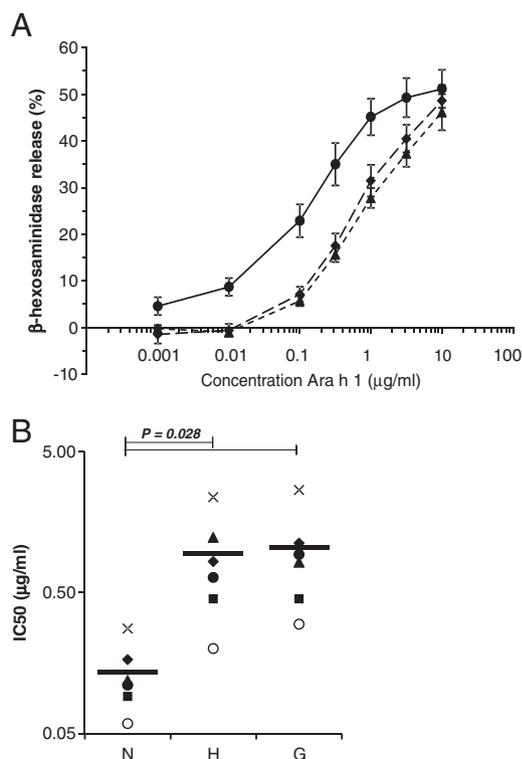
ular or comprised slightly shorter rods with significantly lower heights of around 7–8 nm.

Boiled H- and G-Ara h 1 had greatly reduced IgE-binding capacities for all patient sera analyzed using reverse EAST inhibition, although the pattern of reactivity was hetero-



**Figure 4.** IgE-binding capacity of native and heat-treated Ara h 1. (A and B) IgE capture inhibition curves obtained for serum #2205 and serum #66, respectively, with N-Ara h 1 (○), H-Ara h 1 (◆) or G-Ara h 1 (▲). (C) Analysis of  $IC_{50}$  ( $\mu\text{g}/\text{mL}$ ) values obtained using IgE capture inhibition assay with N-, H- G- and R-Ara h 1 as competitors and labeled N-Ara h 1 as a tracer. A serum panel from 26 peanut allergic patients was used with a reverse EAST inhibition assay. Horizontal bars represent the median  $IC_{50}$  values and are inversely proportional to IgE-binding capacity. Bars indicate a significant difference between the two corresponding Ara h 1 samples determined using a non-parametric Wilcoxon signed rank test. \* $0.01 < p < 0.05$ , \*\* $0.001 < p < 0.01$ , \*\*\* $p < 0.0001$ . Individual symbols represent serum samples from individuals #55 (○), #65 (■), #66 (◆), #70 (▲) and #73 (×) also used in the RBL assay and who also donated PBMC samples.

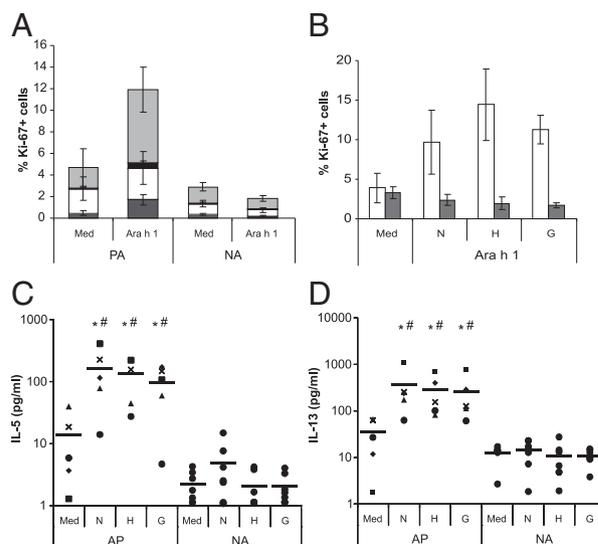
geneous (Fig. 4). Typically, boiling resulted in either a very slight (#2206, Fig. 4A) or more marked (#66, Fig. 4B) reduction in IgE-binding capacity of Ara h 1. This was reflected in the increase in  $IC_{50}$  values for H-Ara h 1 compared with N-Ara h 1 of 1.5- to 5800-fold (mean = 715) and 1.8 to 10 000-fold (mean = 1236) for G-Ara h 1 (Fig. 4C).  $IC_{50}$  values were higher for H- than for G-Ara h 1, for 5 out of 26 sera, showing for these patients that H-Ara h 1 was slightly, but significantly, less IgE reactive, whilst the



**Figure 5.** Effect of thermal treatment on  $\beta$ -hexosaminidase release from humanized rat basophilic leukemia (RBL) cells. (A) Humanized RBL-2H3 cells were passively sensitized with sera from peanut allergic patients (serum #66) and stimulated with increasing concentrations of native (N; —●—), heated (H; --◆-- ) or glycosylated (G; -▲-) Ara h 1. Error bars represent the SD of triplicate values. No  $\beta$ -hexosaminidase release was induced by the different allergens when using sera from non-allergic patients (data not shown). (B)  $\text{IC}_{50}$  values ( $\mu\text{g/ml}$ ) obtained by MRA using six sera from peanut-allergic patients: #55 (○), #65 (■), #66 (◆), #67 (●), #70 (▲) and #73 (×). Horizontal bars represent the means of the six PA subjects. Bars indicate a significant difference between the two corresponding treatments.

remaining 19 sera showed the opposite pattern of reactivity. Remarkably, the IgE immunoreactivity of the R-Ara h 1 was comparable with that of N-Ara h 1, with mean  $\text{IC}_{50}$  values of 31.6 and 37.3 ng/mL, respectively, and showed less inter-individual variability than the N-Ara h 1 (Fig. 4C). Similar patterns of reactivity were observed when following  $\beta$ -hexosaminidase release with a humanized RBL cell line passively sensitized with sera from six patients (Supporting Information Table S1; Figs 4B and 5A, serum #66). However, the loss of reactivity of the H- and G-Ara h 1 was smaller than that observed by EAST, the  $\text{EC}_{50}$  values being increased 6.9- and 7.5-fold, respectively, for H-Ara h 1 and G-Ara h 1 compared with N-Ara h 1 (Fig. 5B).

In addition, the impact of the thermal modifications of Ara h 1 on the T-cell responses of PBMC from peanut allergic patients was assessed. Multicolor FACS staining



**Figure 6.** Effect of thermal treatment on subpopulation of proliferating cells and cytokine induction capacity. (A) Fresh PBMCs were left unstimulated (Med) or stimulated with N-Ara h 1 for 7 days. After harvesting, cells were incubated with anti-CD4/CD25 antibody and afterwards stained for an anti-Ki-67 PE antibody. Data are mean percentage of Ki-67-positive cells  $\pm$  SEM values for 3 (#65, #66 and #70) of the 12 PA subjects and 7 NA controls. The percentage Ki-67<sup>+</sup> cells is divided in the following subpopulations of cells: CD4<sup>-</sup>CD25<sup>-</sup> (dark gray bars), CD4<sup>+</sup>CD25<sup>-</sup> (white bars), CD4<sup>-</sup>CD25<sup>+</sup> (black bars) and CD4<sup>+</sup>CD25<sup>+</sup> (light gray bars) cells. (B) Fresh PBMCs were left unstimulated (Med) or stimulated with N-, H- or G-Ara h 1 for 7 days. Data are mean percentage of Ki-67<sup>+</sup> cells  $\pm$  SEM values for three peanut-allergic patients (65, 66 and 70) (open bars) and seven non-allergic controls (closed bars). Fresh PBMCs were left unstimulated (Med) or stimulated with N-, H- or G-Ara h 1, and production of IL-5 (C) and IL-13 (D) was measured after 7 days of culture. Symbols represent peanut-allergic patients #65 (■), #66 (◆), #70 (▲), #67 (●) and #73 (×). Horizontal bars represent the means of the five PA subjects or the seven NA controls. Asterisks indicate statistically significant differences between the PA and the NA group, and indicates statistically significant difference between the medium and allergen-stimulated cultures for the PA group (\*,  $p < 0.05$ ). No differences between the medium and allergen-stimulated cultures were observed for the NA group.

assessing surface marker expression in combination with intracellular Ki-67 expression as a sensitive method to detect antigen-specific proliferation [18] enabled us to measure subsets of proliferating cells. Stimulation of PBMC cultures from NA controls by Ara h 1 did not induce significant proliferation [ $2.9 \pm 0.8$  and  $1.9 \pm 0.5\%$  for unstimulated (medium) and allergen-stimulated (Ara h 1) cultures, respectively; Fig. 6A]. Only three of the 12 PA subjects showed detectable numbers of Ki-67<sup>+</sup> proliferating cells in the Ara h 1 stimulated cultures (subjects #65, #66 and #70; Fig. 6A) with a corresponding mean percentage of Ki-67<sup>+</sup> cells of  $4.7 \pm 2.7$  for medium and  $11.9 \pm 3.4\%$  for the Ara h 1 stimulation. Proliferating cells consisted of 81% CD4<sup>+</sup> cells,

and the CD4<sup>+</sup>CD25<sup>+</sup> subset of cells was the largest cell population present in the proliferating cell fraction. For these three PA subjects, thermal treatment of N-Ara h 1 had no effect on its capacity to induce PBMC proliferation (Fig. 6B). Whilst no cytokine production was induced by Ara h 1 in PBMC cultures from the NA subjects (Fig. 6C and D; and data not shown), the production of IL-5 and IL-13 was significantly enhanced in the culture of PBMC from 5 out of 12 PA subjects (patients #65, #66, #67, #70 and #73) upon stimulation with N-Ara h 1. Heating in the presence or absence of glucose had no effect on these cytokine secretions. In PA subjects, an enhanced production (compared with NA subjects) was also observed for IL-10 and IFN- $\gamma$ , albeit not significant for all stimuli tested (data not shown). IL-17 production was below 20 pg/mL for all tested conditions (data not shown).

#### 4 Discussion

We have sought to overcome the poor solubility and stability of thermally unfolded and aggregated proteins [2, 9, 17] by using a combination of model processing of purified allergens and purification of allergens from thermally processed peanuts. For the first time, we have shown that boiled Ara h 1 forms complex branched aggregates which have a much reduced IgE-binding capacity. The aggregates also had a reduced capacity to elicit histamine release, a measure of functional biological activity more indicative of a potential reduced allergenic potency in vivo. However, this reduction was not as marked as might be expected given the reduction in IgE-binding capacity, and may reflect the likely multiple IgE epitopes present on the aggregates which might be more efficient for the cross-linking of surface-bound IgE. Further studies in vivo will be required to confirm the potential clinical relevance of this observation.

By comparison, Ara h 1 purified from roasted peanuts formed aggregates which were smaller and morphologically distinct from those induced by boiling and appeared to retain more native-like  $\beta$ -sheet structures by FT-IR than the boiled protein. Such structural features account for R-Ara h 1 retaining its allergenic IgE-binding capacity and are consistent with previous observations that Ara h 1 purified from peanuts heated up to 140°C retained its IgE-binding capacity [9]. These data suggest that aggregate morphology could play a role in affecting IgE binding by altering Ara h 1 epitope availability, a greater proportion of epitopes being masked in aggregates formed by boiling of, compared with the smaller, less complex aggregates adopted by the R-Ara h 1 from roasted peanuts. The formation of branched aggregates with reduced IgE-binding capacity following boiling supports the proposition that boiling reduces the allergenicity of peanut kernels and suggests that many Ara h 1 epitopes are found on the surface of the native proteins and conformational in nature [2].

The ability of Ara h 1 to retain, or even have enhanced allergenicity following roasting, has also been attributed to Maillard modifications. However, the R-Ara h 1 was not as extensively glycosylated as the model processed G-Ara h 1 suggesting that roasting may cause other, uncharacterized modifications of the protein which contribute to its allergenic activity. This may include modification of amino acid residues, such as lysoalanine formation, or adducts formed with other peanut constituents such as lipids. Further characterization of processing induced modification of this protein from roasted peanuts will be required to fully understand what contributes to the ability of this protein to retain its allergenic activity after processing especially since only fructosamine content was measured, which whilst an important intermediate of the Maillard reaction, represents only one of several Maillard adducts that could be formed.

In contrast, the ability of Ara h 1 to stimulate the proliferative activity of PBMC from peanut allergic patients was unaltered following heat treatment and/or glycation, as has been shown for Bet v 1-related allergens [19, 20]. The peanut allergic patients who showed an Ara h 1 specific T-cell response in this study were all characterized by high peanut specific IgE. This suggests that peanut allergic individuals who have high peanut-specific IgE titers have a higher prevalence of allergen-specific T cells and therefore give the most pronounced responses in primary PBMC cultures. However, Turcanu et al. observed that peanut-specific T-helper cells in PBMC of peanut allergic patients were 0.6% of the total CD4<sup>+</sup> T cells in peanut allergic patients and about 10 times lower in non-allergic and peanut sensitized donors [21]. Altogether, these results may explain the low rate of responders in our ex vivo stimulation assays.

Studies of other allergens, particularly in the search for hypoallergenic allergens support the view that aggregation of proteins may affect the quality of immune responses. Recent studies have shown that aggregation may play an important role in establishing protective antibody titers in immunotherapy for the Bet v 1 allergens [22]. The aggregates produced by boiling Ara h 1 presented here resemble the properties of allergoids which have been well accepted for immunotherapy for many years and tend to have lower IgE reactivity, whilst retaining their ability to activate T cells [23]. Boiling for 15 min did not completely abrogate IgE reactivity; however, it may be possible to reduce it further through application longer or of higher temperature wet-heating regimes thus supporting the premise that boiling, as opposed to roasting, may reduce the allergenicity of peanut. Our results also suggest that aggregated forms of allergens induced by physical processes like boiling may also provide a novel form of antigen for use in allergen-specific immunotherapy.

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