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Influence of Thermal Processing on the Allergenicity of Peanut Proteins

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Peanuts are one of the most common and severe food allergens. Nevertheless, the occurrence of peanut allergy varies between countries and depends on both the exposure and the way peanuts are consumed. Processing is known to influence the allergenicity of peanut proteins. The aim of this study was to assess the effect of thermal processing on the IgE-binding capacity of whole peanut protein extracts and of the major peanut allergens Ara h 1 and Ara h 2. Whole proteins, Ara h 1, and Ara h 2 were extracted and purified from raw, roasted and boiled peanuts using selective precipitation and multiple chromatographic steps, and were then characterized by electrophoresis and mass spectrometry. The immunoreactivity of whole peanut extracts and purified proteins was analyzed by the enzyme allergosorbent test (EAST) and EAST inhibition using the sera of 37 peanut-allergic patients. The composition of the whole protein extracts was modified after heat processing, especially after boiling. The electrophoretic pattern showed protein bands of low molecular weight that were less marked in boiled than in raw and roasted peanuts. The same low-molecular-weight proteins were found in the cooking water of peanuts. Whole peanut protein extracts obtained after the different processes were all recognized by the IgE of the 37 patients. The IgE-binding capacity of the whole peanut protein extracts prepared from boiled peanuts was 2-fold lower than that of the extracts prepared from raw and roasted peanuts. No significant difference was observed between protein extracts from raw and roasted peanuts. It is noteworthy that the proteins present in the cooking water were also recognized by the IgE of peanut-allergic patients. IgE immunoreactivity of purified Ara h 1 and Ara h 2 prepared from roasted peanuts was higher than that of their counterparts prepared from raw and boiled peanuts. The IgE-binding capacity of purified Ara h 1 and Ara h 2 was altered by heat treatment and in particular was increased by roasting. However, no significant difference in IgE immunoreactivity was observed between whole protein extracts from raw and roasted peanuts. The decrease in allergenicity of boiled peanuts results mainly from a transfer of low-molecular-weight allergens into the water during cooking.

KEYWORDS: Thermal processing; peanut allergens; allergenicity

BACKGROUND

Peanut allergy is one of the most common and severe IgE-mediated reactions to food because of its severity and lifelong persistence (*1*, *2*). The prevalence of peanut allergy has been estimated between 0.6% and 1% of the U.S. and EU populations and seems to have increased during the past decade (*3*−*5*). In contrast, sensitization and reactivity to peanut is far less prevalent in China, despite the high rate of peanut consumption. It was shown that the Chinese method of cooking (i.e., boiling) and eating peanuts reduces their allergenicity as compared with roasting, which predominates in the U.S. (*6*).

Thermal processing may alter, i.e., increase or decrease, the allergenicity of a protein, but the overall effect on a complex food allergen cannot be predicted (*7*, *8*). The mechanism depends on both the structure and chemical properties of the allergen, the thermal processing used, e.g., dry vs wet, the temperature, and the duration of heating. In addition, interactions with other constituents of the food matrix may occur and have a major effect on the overall allergenicity of the food (*9*). Pastorello et al. (*10*) did not observe any loss of IgE-binding capacity in a lipid transfer protein (LTP) of maize, after a thermal treatment at 100 °C for 160 min. Wigotzki et al. (*11*)

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also showed that dry processing at 100 °C for up to 90 min had no effect on the allergenicity of some hazelnut proteins and suggested the occurrence of very heat stable allergenic proteins with molecular weight less than 14 kDa. However, the IgE-binding capacity of other hazelnut allergens was decreased after 15 min of heat treatment at a temperature between 100 and 185 °C. According to Hansen et al. (*12*), roasting for 40 min at 140 °C led to a decrease in allergenicity of the birch-pollen-related allergens Cor a 1.04 and Cor a 2 but increased the allergenicity of the LTP Cor a 8. This is of major importance for the management of hazelnut allergy in people of Central and Northern Europe mainly sensitized to Bet v 1 related allergens or in people of Southern Europe who are mainly sensitized to LTP (*12*).

Among the different peanut proteins, some are well-characterized allergens, e.g., the 7S globulin Ara h 1 and the 2S albumin Ara h 2 (*13*, *14*). Peanut allergens are mostly seed storage proteins. Roasting treatment enhances IgE-binding capacity (*15*). Unlike roasting, boiling decreases peanut allergenicity (*6*). The effect of thermal processing on whole peanut immunoreactivity results from modification of the structure and reactivity of each individual allergen and of their interaction with the food matrix.

The aim of this study was to assess changes in the IgE-binding capacity of whole peanut proteins and of the two major peanut allergens Ara h 1 and Ara h 2 due to thermal processing such as roasting and boiling in comparison with raw peanut.

MATERIALS AND METHODS

Human Sera. Thirty-seven patients were recruited at the paediatric allergy clinic of the Hôpital Necker-Enfants Malades, Paris, France. All the patients had a confirmed peanut allergy on the basis of an extensive history, physical examination, skin prick testing, and objective manifestations observed after peanut ingestion. The study population was mainly composed of children (mean age 8 years, median 5 years): no patient was under 2 years old, 9 patients were between 2 and 4 years old, 10 patients were between 4 and 6 years old, 2 patients were between 6 and 8 years old, 3 patients were between 8 and 10 years old, and 13 patients were over 10 years old. All the patient sera were individually tested.

Reagents. Unless otherwise stated, all reagents were of analytical grade and obtained from Sigma (St. Louis, MO). Whole peanut proteins and pure allergens were prepared using 20 mM phosphate, pH 7.4, 1 M NaCl buffer for extraction and 20 mM phosphate, pH 7.4, buffer for dialysis.

Buffers and reagents used for Western blotting were as follows: TBS: 20 mM Tris, pH 8.0, 0.25 M NaCl; TBST: 20 mM Tris, pH 8.0, 0.25 M NaCl, 0.5% Tween. Buffers and reagents used for enzyme immunoassay (EIA) were as follows: EIA buffer: 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1% BSA (bovine serum albumin), 0.4 M NaCl, 1 mM EDTA, and 0.01% sodium azide; washing buffer: 0.01 M potassium phosphate buffer, pH 7.4, containing 0.05% Tween 20; Ellman reagent: 7.5×10^{-4} M acetylthiocholine and 5×10^{-4} M dithio-bis-nitrobenzoate in 100 mM potassium phosphate buffer, pH 7.4.

Solid-phase enzyme immunoassays were performed in 96-well microtiter plates (Nunc, Denmark) using automatic Titertek microtitration equipment (washer, dispenser, and reader) from Labsystems (Helsinki, Finland).

Preparation of Whole Peanut Protein Extracts and Purified Allergens. *Whole Peanut Protein Extract (WPPE)*. Raw and commercially roasted peanuts (Virginian variety) were used. Kernels of raw peanuts were boiled for 30 min in water. After cooking, the water (water PE) was collected and used for further analysis of the protein content. After each treatment, peanut kernels were peeled and ground until a homogeneous paste was obtained. The paste was defatted using 5 volumes (w/v) of cold ether and dried overnight under nitrogen flow at room temperature. Proteins were extracted by stirring in extraction buffer (10% w/v) overnight at 4 °C. After centrifugation at 4000*g* for 20 min at 4 °C, the supernatant was collected and the pellet was suspended in 4 M urea for 4 h at room temperature. After a second centrifugation under the same conditions, the 4 M urea supernatant was collected and pooled with the previous one. The pooled supernatants of the different extracts were then dialyzed against dialysis buffer, and dialyzed extracts from raw (raw PE), roasted (roasted PE), and boiled (boiled PE) peanuts were stored at −80 °C. An aliquot of the raw PE was heat-treated by boiling for 30 min. Protein concentration in the extracts was determined using the BCA method (Pierce).

Purified Allergens. Ara h 1 and Ara h 2 were prepared and purified from raw, roasted, and boiled PE (i.e., raw, roasted, and boiled Ara h 1 or Ara h 2). Each of the three extracts was precipitated using 40% ammonium sulfate. After centrifugation, the pellets were discarded and the supernatants were dialyzed.

Ara h 1 was then separated and purified by a combination of chromatographic methods including the following: 1/affinity chromatography on a Con A Sepharose column, equilibrated with 40 mM Tris, pH 7.4, NaCl 0.5 M buffer, elution 40 mM Tris, pH 7.4, 0.5 M NaCl, 0.5 M methyl α,D-mannopyranoside at 1 mL/min; 2/RP HPLC on an AKTA purifier system (Pharmacia) on a C4 (Vydac 250 × 10 mm) column, equilibrated with H_2O, 0.1% trifluoroacetic acid (TFA), elution acetonitrile, 0.04% TFA, at 8 mL/min.

Ara h 2 was isolated from the unbound fraction of the Con A Sepharose affinity chromatography. This fraction was dialyzed. After addition of 4 M urea, the dialyzed fraction was first purified by anion exchange chromatography using a 30Q Sepharose column, equilibrated with 50 mM Tris, pH 8.0, 4 M urea, elution 1 M NaCl at 8 mL/min. Purification was then achieved using the same reversed-phase chromatography as for the purification of Ara h 1.

Ara h 1 and Ara h 2 were characterized by electrophoresis, MALDI-TOF mass spectrometry analysis using a Voyager DE RP apparatus (PE Biosystem), and N-terminal amino acid sequence analysis using the Edman method.

Electrophoresis. Whole peanut protein extracts and purified allergens were analyzed using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) under reducing conditions (2.5% β-mercaptoethanol) as described by Laemmli (*16*) and modified by Dean et al. (*17*).

Western Blotting. After electrophoresis, proteins were transferred to PVDF membranes (Immobilon Millipore, Polylabo) for 90 min at 36 V using a MiniProtean III system (BioRad). The membranes were saturated with TBST supplemented with 5% milk powder. Allergic patient sera diluted in the same buffer were incubated overnight at 4 °C. Membranes were then washed, and a human anti-IgE monoclonal antibody labeled with peroxidase (MCA 2116P, Serotec) was incubated for 1 h at room temperature. After several washings, the membrane was incubated with the Supersignal West Dura extended duration substrate (PIERCE) for 1 min and then revealed on X-OMAT films (Kodak).

Determination of Specific IgE Responses to the Whole Peanut Protein Extracts. The IgE-binding capacity of the different extracts was analyzed using the enzyme allergosorbent test (EAST) previously described. Microtiter plates were coated with the different whole peanut protein extracts (i.e., raw, roasted, boiled, and water PE) at a concentration of 10 μg/mL. Serial dilutions of 100 μL of each serum were dispensed per well and incubated for 24 h at 4 °C. An anti-human IgE antibody (BS17 clone) labeled with acetylcholinesterase (AChE) was used as a tracer. Ellman's reagent was used as an enzyme substrate. Specific IgE were quantified by comparison with concentration−response curves obtained with a total IgE assay performed under identical conditions using a solid phase coated with a second anti-human IgE antibody (LE27) instead of peanut proteins, which is complementary to BS17-AChE tracer (*18*−*20*).

Analysis of the Immunoreactivity of the Whole Peanut Extracts. EAST inhibitions for raw, roasted, boiled, and water PE were performed as described above using five sera representative of the study population, except that a preliminary step was added; i.e., 40 μL samples of allergic patient sera were first preincubated with 40 μL of inhibitor solution (i.e., peanut protein extract) for 4 h at room temperature. A 50 μL

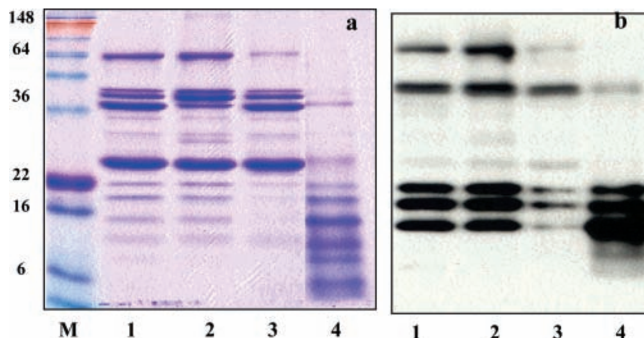

Figure 1. (**a**) Electrophoretic pattern of the different whole peanut extracts: (1) raw PE, (2) roasted PE, (3) boiled PE, and (4) water PE. Molecular weight markers are loaded on the well marked "M". (**b**) Western blotting performed on whole peanut extracts with a serum representative of our study population: (1) raw PE, (2) roasted PE, (3) boiled PE, (4) water PE.

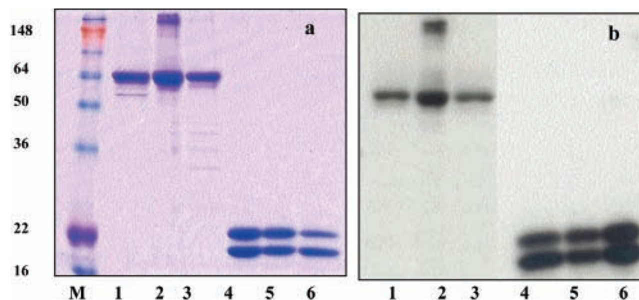

Figure 2. (**a**) Electrophoretic pattern of the different purified proteins: (1) raw Ara h 1, (2) roasted Ara h 1, (3) boiled Ara h 1, (4) raw Ara h 2, (5) roasted Ara h 2, and (6) boiled Ara h 2. Molecular weight markers are loaded on the well marked "M". (**b**) Western blotting performed on the different purified proteins with a serum representative of our studied population: (1) raw Ara h 1, (2) roasted Ara h 1, (3) boiled Ara h 1, (4) raw Ara h 2, (5) roasted Ara h 2, and (6) boiled Ara h 2.

sample of the serum/inhibitor mix was then dispensed per well on a microtiter plate coated with raw PE. Inhibitor solutions contained increasing concentrations of raw, roasted, boiled, or water PE (from 1 ng/mL to 100 μg/mL).

Results were expressed as B/B_0, where B_0 and B correspond to the specific IgE binding to immobilized raw PE in the absence or presence of a known concentration of inhibitor, respectively. Concentrations of the different peanut extracts that inhibit 50% of the IgE binding to coated raw PE (IC_{50}) were determined for each individual patient serum.

Analysis of the Immunoreactivity of the Purified Allergens. The immunoreactivity of purified Ara h 1 and Ara h 2 was analyzed using the same five sera. EAST inhibition studies were performed on microtiter plates coated with a monoclonal anti-human IgE antibody (i.e., LE27). Dilutions of 100 μL of each serum were distributed per well and incubated overnight at 4 °C. After washing, 50 μL of inhibitor and 50 μL of tracers were dispensed and incubated for 4 h at room temperature. Inhibitors consisted of increasing concentrations of raw, roasted, and boiled Ara h 1 or Ara h 2. Enzymatic tracers were prepared by covalent linkage of Ara h 1 or Ara h 2 to the tetrameric form of AChE as previously described for other protein tracers (*21*).

After washing, Ellman's reagent was used as an enzyme substrate. Results were expressed as B/B_0, where B_0 and B represent the amount of Ara h 1 or Ara h 2 tracers linked to immobilized IgE in the absence or presence of a known concentration of inhibitor, respectively.

Statistical Analysis. Due to a non-Gaussian distribution of specific IgE levels, means and medians were analyzed using nonparametric statistical methods (Friedman and Spearman rank correlation tests).

RESULTS

Composition of Whole Peanut Protein Extracts. The same extraction method was applied to the analysis of raw, roasted, and boiled PE. After solvent defatting and aqueous extraction, 706 mg of protein was obtained from 5 g of raw and roasted peanut kernels. On the basis of a 25% protein content in peanut kernels (*22*), the extraction yield was near 60% in both raw and roasted peanuts. In contrast, only 377 mg of proteins was obtained from 5 g of boiled peanut kernels, e.g., a 2-fold lower recovery. Moreover, proteins were found in the water used to cook the peanuts. The protein concentration in the water was 39 mg in 100 mL (i.e., the volume used to cook 10 g of peanut kernels).

The protein compositions of raw, roasted, boiled, and water PE were compared using SDS−PAGE under reducing conditions (**Figure 1a**). All the analyzed samples were at the same concentration (i.e., 1 mg/mL). Similar patterns were observed in raw PE, roasted PE, and boiled PE including a band of ca. 65 kDa molecular weight (MW) corresponding to Ara h 1, several bands ranging from 25 kDa to 45 kDa MW, which very likely correspond to glycinin fragments, and two bands at ca. 16 kDa and 18 kDa MW corresponding to the two characteristic isoforms of Ara h 2. It is noteworthy that Ara h 1 and Ara h 2 concentrations in boiled PE were much lower than in raw and roasted PE. In addition, low-MW protein bands ranging between 10 kDa and 16 kDa, which correspond to 2S albumins or peptide fragments (MW < 1000 Da), were still present in boiled PE, although in lower amounts than in raw and roasted PE. The cooking water electrophoretic pattern shows the presence of proteins and particularly of Ara h 2 and of those low-MW proteins whose concentration was decreased in boiled PE.

Figure 2a shows the analysis of Ara h 1 and Ara h 2 that were extracted and purified from raw, roasted, and boiled PE. The MWs determined by mass spectrometry analysis and N-terminal amino acid sequencing were identical in the three cases. Ara h 1 always appeared as a single band close to 65 kDa MW. A band of high MW (over 148 kDa) can be observed in roasted Ara h 1. This band is very likely due to the trimeric form of Ara h 1 as already described by Maleki et al. (*15*) and Beyer et al. (*6*). In every case, Ara h 2 presented two bands of ca. 16 kDa and 18 kDa MW, corresponding to the two isoforms.

Western Blotting. Western blotting was performed using three sera, numbers 23, 31, and 37. All patterns were identical to those shown in **Figures 1b** and **2b** using serum 23.

Figure 1b shows the allergenic proteins contained in raw, roasted, boiled, and water PE. In all the extracts, the same allergens were present, i.e., Ara h 1 (MW of ca. 65 kDa), glycinin fragments (MW ranging between 25 kDa and 45 kDa), Ara h 2 (two isoforms of MW of 16 kDa and 18 kDa), and proteins of low MW ranging from 10 kDa to 16 kDa very likely represented by 2S albumins and fragments thereof.

In boiled PE, the concentration of Ara h 1, Ara h 2, and other 2S albumin bands was lower than that observed in raw and roasted PE.

Purified Ara h 1 and Ara h 2 were recognized by specific IgE in the same manner regardless of which of the three extracts (raw, roasted, and boiled PE) they were prepared from (**Figure 2b**). The trimeric form of Ara h 1 was present in the roasted Ara h 1 and gave a band of high molecular weight well recognized by specific IgE.

Determination of the Specific IgE Response to the Whole Peanut Protein Extracts. For each patient, the determinations of specific IgE to raw, roasted, boiled, and water PE are presented in **Table 1**. **Figure 3** shows marked heterogeneity in the intensity of the specific IgE responses. As shown in **Figure 3**, mean or median values of IgE responses to raw and roasted

Table 1. IgE Specific Responses of the 37 Sera Expressed in IU/mL

serum	specific IgE response: extract from raw peanut	extract from roasted peanut	extract from boiled peanut	extract from cooking water
1	4	3	2	2
2	5	5	1	1
3	7	8	2	2
4	17	18	11	12
5	39	41	25	23
6	5	7	1	1
7	11	16	5	5
8	11	10	6	3
9	42	45	18	24
10	22	26	13	15
11	29	29	16	16
12	20	21	8	9
13	27	27	21	22
14	213	250	153	133
15	189	210	127	111
16	258	293	172	200
17	66	176	33	26
18	118	276	57	48
19	192	149	110	60
20	219	221	145	135
21	162	201	109	103
22	2382	2900	1445	1325
23	487	586	259	153
24	144	135	92	98
25	361	439	242	240
26	498	456	306	290
27	189	226	143	135
28	131	112	108	73
29	39	60	20	23
30	742	693	426	377
31	399	453	200	176
32	1016	1331	705	632
33	296	288	152	95
34	124	139	63	59
35	671	628	352	317
36	792	756	586	410
37	1138	1150	705	429

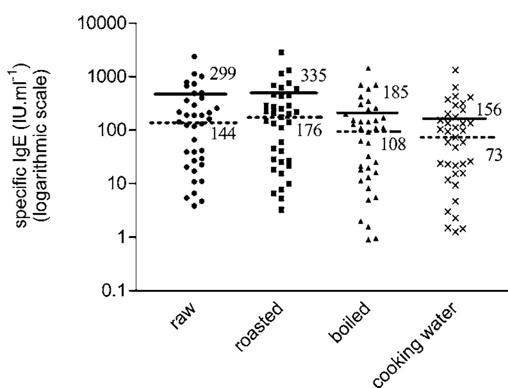

Figure 3. Specific IgE response to the different whole peanut protein extracts. Each point represents the concentration of specific IgE expressed in IU/mL for each of the 37 tested sera: —, mean value for the 37 sera; ---, median value for the 37 sera.

PE were very similar and the IgE response to water PE had the lowest mean and median values. Median values of IgE titers against boiled PE were approximately 1.5−2-fold lower than those against raw and roasted PE. Note that the sum of the median IgE titers against boiled and water PE ($\Sigma = 181$ IU/mL) was close to the IgE responses to raw PE (144 IU/mL) and roasted PE (176 IU/mL). **Table 2** shows the statistical analysis using a nonparametric test (Friedman test). There were no significant differences between IgE titers against raw and roasted PE. IgE responses to raw and roasted PE were significantly higher ($P < 0.001$) than those to boiled or water PE. Differences between IgE levels in boiled and water PE were also significant ($P < 0.05$) (**Table 2**).

The estimated values for the Spearman rank correlation showed a high correlation for each pair (**Table 2**). Values were always higher than 0.97 and highly significant even when comparison implied levels of IgE to water PE.

Table 2. Statistical Analysis of the Specific IgE Response to the Whole Peanut Protein Extracts after the Different Heat Treatments of the Peanuts (Friedmann Test and Spearman Rank Correlation)[a]

		roasted vs raw	roasted vs boiled	raw vs boiled	roasted vs water	raw vs water	boiled vs water
Friedmann test	P	ns	***	***	***	***	*
Spearmann correlation	r	0.99	0.98	0.99	0.98	0.99	0.99
	P	***	***	***	***	***	***

[a] *r* represents the coefficient of correlation, and *P* represents the probability to obtain a significant effect (*, $P < 5\%$, significant test; **, $P < 1\%$, very significant test; ***, $P < 10^{-3}$, highly significant test; ns, nonsignificant).

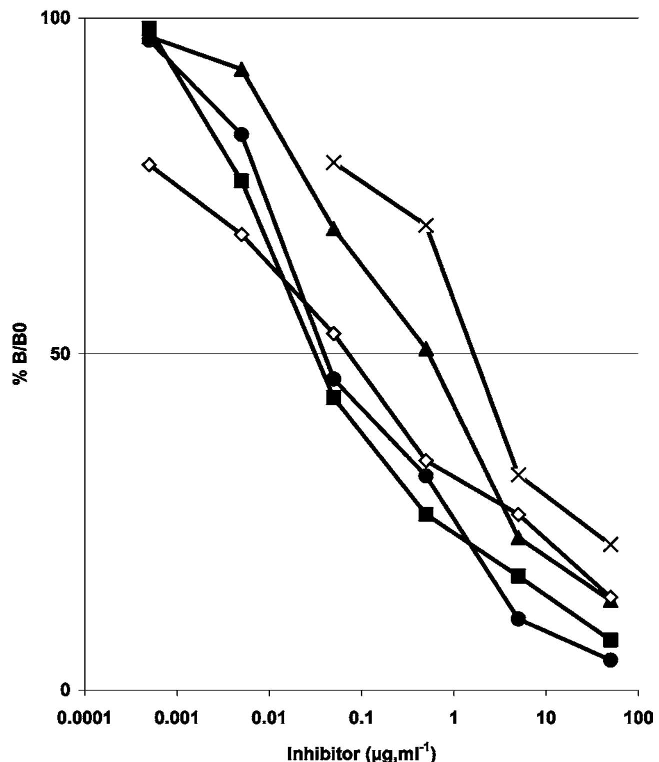

Figure 4. Inhibition of the IgE binding to immobilized raw PE by increasing concentrations of whole peanut extracts using serum 21: ■, raw PE; ●, roasted PE; ▲, boiled PE; ×, water PE; ◇, boiled raw PE.

Analysis of the Immunoreactivity of the Whole Peanut Protein Extracts. Immunoreactivity of the different peanut protein extracts was analyzed by EAST inhibition. **Figure 4** shows inhibition curves of IgE binding to raw PE obtained for patient serum 21 by increasing concentrations of raw, roasted, boiled, and water PE. The curves are representative of those observed with the different sera. The IgE binding was partially or completely inhibited by each of the different peanut protein extracts and by the raw PE that was boiled for 30 min. Inhibition curves obtained with raw and roasted PE were similar. Boiled PE was a weaker competitor. IC_{50} values measured with boiled PE were 10−50-fold higher than those of raw and roasted PE. Interestingly, inhibitions obtained with raw PE heat-treated (i.e., boiled) after extraction were very close to those obtained with

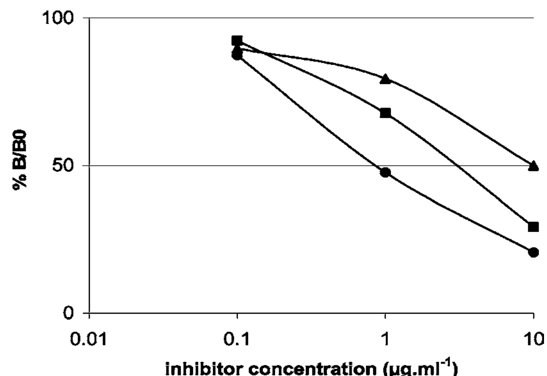

Figure 5. Inhibition of the binding of immobilized IgE to Ara h 1 tracer by increasing concentrations of Ara h 1 purified from different extracts, using serum 21: ●, Ara h 1 purified from roasted PE; ■, Ara h 1 purified from raw PE; ▲, Ara h 1 purified from boiled PE.

raw PE itself and higher than those obtained with boiled PE. The protein extract from the cooking water gave parallel inhibition curves although the IC_{50} was about 5-fold higher than with boiled PE.

Results presented above and in **Figure 4** were obtained with immobilized raw PE. They were the same for IgE binding to immobilized roasted PE and its inhibition by the different extracts.

Analysis of the Immunoreactivity of the Purified Allergens. The immunoreactivity of purified Ara h 1 and Ara h 2 from raw and heat-processed peanuts was analyzed by competitive EIA. Increasing concentrations of purified raw, roasted, and boiled Ara h 1 (respectively Ara h 2) were used to inhibit the binding of Ara h 1−AChE (respectively Ara h 2−AChE) tracers to immobilized allergic patient serum IgE.

Raw, roasted, and boiled Ara h 1 or Ara h 2 IC_{50} values were measured for each individual serum of five representative patients (i.e., patients 12, 21, 25, 28, and 30) of the study population.

The binding of Ara h 1−AChE to IgE was inhibited by any of the raw, roasted, and boiled Ara h 1. With all the patients, the highest inhibitions were observed with roasted Ara h 1 as shown for one serum (21) in **Figure 5**. Roasted, raw, and boiled Ara h 1 IC_{50} values ranged from 0.3 to 1.5 μg/mL (i.e., from 5 to 25 pmol/mL), from 1.5 to 10 μg/mL (i.e., from 25 to 160 pmol/mL), and from 0.9 to 10 μg/mL (i.e., from 15 to 160 pmol/mL), respectively. When the ratios of IC_{50} of roasted to raw Ara h 1 and of roasted to boiled Ara h 1 were considered, the values obtained for each serum ranged from 1/2 to 1/20 and from 1/3 to 1/20, respectively. For four sera out of five, raw Ara h 1 showed a stronger inhibition than boiled Ara h 1 with an IC_{50} ratio of about 1/3. For one serum, the IC_{50} values of raw and boiled Ara h 1 were similar.

For all the patients, the binding of Ara h 2−AChE to immobilized IgE was also completely inhibited by raw, roasted, or boiled Ara h 2. The highest inhibitions were observed with roasted Ara h 2 as shown in **Figure 6** for serum 21. Roasted, raw, and boiled Ara h 2 IC_{50} values ranged from 2 to 6 ng/mL (i.e., from 0.1 to 0.3 pmol/mL), from 4 to 15 ng/mL (i.e., from 0.2 to 0.8 pmol/mL), and from 4 to 20 ng/mL (i.e., from 0.2 to 1 pmol/mL), respectively. IC_{50} values measured with raw and boiled Ara h 2 were similar. For the different patients, the ratios of IC_{50} of roasted to either raw or boiled Ara h 2 were about 1/3.

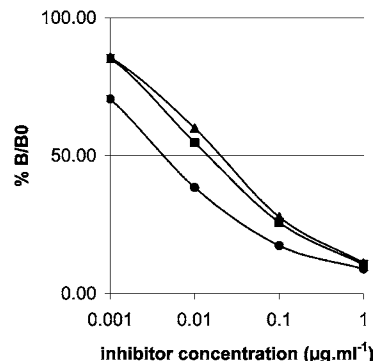

Figure 6. Inhibition of the binding of immobilized IgE to Ara h 2 tracer by increasing concentrations of Ara h 2 purified from different extracts, using serum 21: ●, Ara h 2 purified from roasted PE; ■, Ara h 2 purified from raw PE; ▲, Ara h 2 purified from boiled PE.

DISCUSSION

Significant alterations in protein structure may occur during heat treatments, the nature and extent of which depend on the temperature and the duration of the thermal processing. Typically, loss of tertiary structure is followed by reversible unfolding, loss of secondary structure (70−80 °C), formation of new intra/intermolecular interactions, rearrangements of disulfide bonds (80−90 °C), and formation of aggregates (90−100 °C) (*9*). Alteration of structure may affect allergenicity. Heat treatments can destroy conformational epitopes by denaturation of the proteins and result in the fact that only linear epitopes are available for binding to antibodies (*9*). Denaturation may thus explain the loss of 90% of immunoreactivity of heat-labile birch-pollen-related allergens of hazelnuts such as Cor a 1.04 and Cor a 2 (*12*). However, this phenomenon is not systematically observed and depends on the intrinsic characteristics of the protein. Allergenicity of some small proteins, particularly LTPs, is not affected by thermal treatment at 100 °C for up to 90 min in maize (*10*) and in hazelnut (*12*). Moreover, Maleki et al. (*15*) showed that the IgE-binding capacity of roasted peanuts was approximately 90-fold higher than that of raw peanuts of the same cultivars. Roasting of peanut is usually performed at ca. 140 °C for 40 min. At high temperature, chemical modifications may occur with covalent links between lysine residues of the protein and other constituents of the food matrix, leading to various adducts (*23*). They may contribute to the formation of new immunologically reactive structures. It has also been demonstrated that roasting Ara h 1 forms highly stable trimers. All these modifications could contribute to the increased IgE-binding capacity of roasted Ara h 1 observed in most peanut-allergic patients. Structural modifications in Ara h 2 after roasting observed by Maleki et al. (*15*, *24*, *25*) may also result in an increased IgE-binding capacity. In the present study, we have also observed that purified Ara h 1 and Ara h 2 presented the highest IgE-binding capacity when they were prepared from roasted peanut. However, the differences in immunoreactivity between roasted and raw peanut were much lower than previously described.

Alterations in protein structure and the consequences for their allergenicity could also depend on the conditions of heat processing, e.g., dry vs wet treatment heating, and Beyer et al. (*6*) demonstrated that the allergenicity of peanut was decreased by boiling.

In the present study, we analyzed the impact of some heat treatments on the allergenicity of peanut proteins. The IgE-binding capacity of both the whole food, i.e., whole peanut

proteins, and purified Ara h 1 and Ara h 2 was analyzed after roasting and boiling.

Whole proteins, Ara h 1, and Ara h 2 were prepared from raw, roasted, and boiled peanuts using the same procedure. The protein content was quantified and then analyzed. Ara h 1 and Ara h 2 had the same properties in terms of chromatographic and electrophoretic behavior, N-terminal amino sequence, and mass spectrometry. In particular, Ara h 2 isoforms were observed in raw, roasted, and boiled PE. Protein recovery was similar in raw and roasted PE. After boiling, part of the protein was present in the boiled PE but a part also remained in solution in the cooking water, particularly proteins of low MW.

In the study population, we observed a variability in IgE response to whole peanut protein extracts. However, the same heat processing effects on the IgE-binding capacity of whole peanut protein extracts were observed in all patients. The IgE responses to raw and roasted PE were not significantly different, whereas those to boiled and water PE were approximately 1.5−2-fold lower.

As a result of the non-Gaussian distribution of the specific IgE levels, medians and means were calculated and analyses were performed using nonparametric statistical methods (Friedman test and Spearman rank correlation test). According to the Friedman test, no significant difference was evidenced between raw and roasted PE. Highly significant differences ($P < 0.001$) were evidenced between raw or roasted PE and boiled PE and water PE. A significant difference ($P < 0.05$) was observed between boiled PE and water PE. The Spearman rank correlation test showed a highly significant correlation between the specific IgE responses to the four samples.

In addition to the quantification of specific IgE, the analysis of the effects of heat treatments on the allergenicity of whole peanut proteins by EAST inhibition gave similar information on the apparent affinity of the IgE−peanut protein interaction. Inhibition of IgE binding to immobilized raw PE was similar when raw PE and roasted PE were used as competitors, but the IC_{50} values of boiled and water PE were 10−50-fold higher than those of raw and roasted PE.

Inhibition studies of Ara h 1 and Ara h 2 were performed using an enzyme immunoassay different from classical EAST. An original inhibition test was developed to overcome difficulties in obtaining inhibition curves, particularly with Ara h 2, and to improve the sensitivity and specificity of the test. The difficulties were likely due to the structural polymorphism of Ara h 2 and to the heterogeneity of anti-Ara h 2 specific IgE populations. In this assay, all the IgE's (including total IgE and IgE specific to peanut proteins) were immobilized using anti-human IgE monoclonal antibodies (LE27 done) which were passively adsorbed on the microtiter plates. LE27 did not bind IgG's. IC_{50} values were at least 100-fold lower for Ara h 2 than for Ara h 1, which could suggest that the IgE's of the allergic patients have a higher apparent affinity for Ara h 2 than for Ara h 1. The difference is attenuated if the concentration of inhibitors is expressed in molarity and not in, e.g., mg/mL. Exact comparison between the IC_{50} values of Ara h 1 and Ara h 2 is also complicated by the fact that the preparation of tracers, particularly of Ara h 1−AChE, is not fully controlled in terms of the concentration of activated intermediary derivatives and finally in terms of the enzyme/allergen ratio, which may influence the apparent affinity of specific IgE for Ara h 1 and for Ara h 1−AChE.

Specific anti-Ara h 1 and anti-Ara h 2 IgE's of patient 21 whose serum was used to plot the inhibition curves in **Figures 4−6** were quantified in a previous study and were 176 and 181 IU/mL, respectively (*20*).

Regarding the effect of heat treatments for all the tested sera, Ara h 1 and Ara h 2 purified from roasted PE showed a higher inhibitory capacity than those purified from raw and boiled PE. Interestingly, this difference was no longer apparent with whole food, i.e., whole peanut proteins.

Ara h 2 protects Ara h 1 from degradation by trypsin, and this protective characteristic is enhanced by roasting (*24*). An interaction with Ara h 2 during roasting treatment may explain the enhancement of the immunoreactivity of Ara h 1 from roasted PE. Maleki et al. (*15*) showed that the Maillard reaction products contribute to the increase in IgE-binding capacity of peanut after roasting. However, the increase in immunoreactivity of Ara h 1 from roasted PE they observed was much greater than that observed in the present study. The discrepancy in Ara h 1 (and particularly of "roasted" Ara h 1) reactivity may be due to differences in the study population and particularly in their dietary habits. Exposure mainly to roasted peanuts may be greater for Americans than for European populations. It may facilitate and enhance sensitization to the highly stable trimers of Ara h 1 formed during the roasting process. All these modifications could contribute to greater IgE recognition of roasted Ara h 1 by some patients.

In the case of Ara h 2, inhibition studies showed no difference in immunoreactivity between Ara h 2 from raw or boiled PE and a higher immunoreactivity after roasting. The structure of Ara h 2, a 2S albumin, has similar cysteine motifs to LTP and as a consequence an LTP-like folding (*26*) which results in the heat-stable property of those proteins (*27*). Intramolecular cross-linking caused by roasting (*24*) contributes to the increase in allergenic properties, and it is likely that enhanced trypsin-inhibitory activity also plays a role in this increased allergenicity.

The allergenicity of a whole food depends on the contribution of several allergens that may react differently to processing and to heat treatments. The nature, intensity, length, and conditions of heat treatment may also impact differently the structure of allergenic proteins, their interactions with other constituents of the food matrix, and finally their allergenicity. Some allergenic structures may be destroyed, whereas others, particularly low-molecular-weight peptide fragments, may be newly formed. All those interactions may explain why the effects of heat treatments are eliminated or attenuated for whole peanut food as compared with isolated pure allergens.

The global decrease in allergenicity observed in boiled peanut was not associated with structural modifications of proteins but mainly with a loss of allergens, essentially of low-molecular-weight proteins or peptide fragments from kernels, and their transfer by solubilization into the cooking water. This seems to correlate with the low prevalence of peanut allergy in countries where peanuts are cooked in this way.

Wet process heating (e.g., boiling) could be used in the processing applied in the food industry to decrease the allergenicity of peanut protein fractions used for the preparation of different foods. However, decreasing the allergenicity of whole peanuts is an inadequate solution for the management and prevention of the allergy risk. Our results underline the importance of food product labeling when known allergenic foods such as peanuts are involved in industrial processing.

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