

The effect of cooking methods on the allergenicity of peanut

YAN-JUN CONG¹, FEI LOU¹, WEN-TONG XUE¹, LIN-FENG LI²,
JING WANG², & HUI ZHANG¹

¹College of Food Science & Nutritional Engineering, China Agricultural University, and

²Department of Dermatology, Peking University Third Hospital, PR China

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Abstract

It has been rarely studied why the prevalence of peanut allergy is relative low in China. The aim of this study was to investigate: (i) the major peanut allergens in China, (ii) the effect of buffer composition on peanut allergens extraction efficiency, and (iii) the effect of cooking methods on peanut allergenicity. The allergenic property of peanut protein extracts was assessed by immunoblotting. The relative contents of the major peanut allergens were quantified by densitometry. The ability of IgE to bind to the different peanut preparations (fried, boiled and roasted) was detected with ELISA. In conclusion, Ara h 1 and several proteins belonging to the Ara h 3 were major peanut allergens. Different buffers lead to different extraction efficiency for allergen fragments. The IgE binding property of peanuts processed by cooking, frying and roasting did not differ significantly. Therefore, cooking methods may not explain the reason for the lower prevalence of peanut allergy in China.

Keywords: *Peanut allergens, cooking methods, Ara h 1, buffer composition, allergenicity, peanut proteins.*

Introduction

Eight foods are responsible for more than 90% of food allergies: cow's milk, eggs, soy, cereals containing gluten, peanuts, tree nuts (walnuts, hazelnuts, almonds, Brazil nuts, cashews, pecans and pistachios), fish and shellfish (Hefle et al. 1996). In Europe sesame seed and mustard are also regarded as common food allergens (NDA 2005). Among food allergies, peanuts and peanut-derived products elicit the most severe allergic reactions, often with fatal outcome.

Allergenic components of peanuts have been identified by immunoglobulin E (IgE) binding properties for the following protein sub-units or peptides: Ara h 1 (Burks et al. 1991, 1995, 1997; Rabjohn et al. 1999), Ara h 2 (Burks et al. 1992; De Jong et al. 1998; Rabjohn et al. 1999), Ara h 3 (Koppelman et al. 2003), Ara h 4 (Rabjohn et al. 1999), Ara h 5 (Rabjohn et al. 1999), Ara h 6 (Rabjohn et al. 1999), and Ara h 7 (Rabjohn et al. 1999) and others. The Ara h 1, Ara h 2 and Ara h 3 are major allergens

Correspondence: Dr Wen-Tong Xue, Department of Food Science & Nutritional Engineering, China Agricultural University, 17 Qinghua Eastern Road, Haidian district, Beijing 100083, P. R. China. Tel: +861062736734 81. Fax: +861062736734 84. E-mail cyj_win@sina.com.cn

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because they recognize IgE in more than 70% of the peanut allergy sera. Their molecular weights are 63 kDa, 17–20 kDa, 14–45 kDa, respectively. Peanut allergens are resistant to digestion and heat treatment has no effect on the allergenicity. The estimated prevalence of peanut allergy in developed countries is between 0.6% and 1.0% (Emmett et al. 1999; Kanny et al. 2001; Sicherer et al. 2003). Recently, a follow-up study (Grundy et al. 2002) demonstrated that the prevalence of peanut allergy had increased to 1.5% on the Isle of Wight, which suggests that the problem is growing. This was also reported by Sicherer et al. (2003). In contrast, China has one of the lowest prevalence rates for certain atopic diseases in a worldwide comparison (The International Study of Asthma and Allergies in Childhood [ISAAC] Steering Committee 1998). Up until now, few studies have reported why the prevalence of peanut allergy in China is much lower than the United States despite the high rate of peanut consumption. The aim of this study was to identify the major allergens of raw peanuts in China and study the effect of different cooking method on the allergenicity of peanuts.

Material and methods

Peanut protein extraction

Three commonly consumed peanut varieties in China: Baisha, Luhua and Huayu were kindly provided by Seed Co., of Beijing. Baisha is mainly used in the process of peanut protein containing foodstuffs, Luhua is used in the production of peanut oil, and Huayu is used mainly as an ingredient (additives) in foods.

Peanut seeds were peeled and ground in a mortar with a pestle. Defatting was performed for 4 h. Hexane, instead of petroleum ether, was used as an extraction agent (Basha & Cherry 1976). Defatted peanuts were air-dried overnight. Then, defatted peanut proteins were extracted in a 1 MNaCl, 20 mM sodium phosphate buffer (pH 7.6) in at ratio of 1:20 (w/v) for 4 h at 4°C (Basha & Cherry 1976). The protein extract was clarified by centrifugation at 12,000 *g* for 15 min at 4°C. The supernatant containing the total soluble protein was dialyzed for 48 h in distilled water. Extracts were stored at –20°C until use.

The protein content of crude peanut was determined by the Kjeldahl method, and the protein content of peanut extracts was determined by the Lowry method (Lowry et al. 1951). The protein content of the three peanut varieties Baisha, Luhua and Huayu are 27.38%, 21.06% and 23.90%, respectively. The protein value between different varieties differ thus somewhat. All samples resulted in similar standard deviations.

Peanut preparations

Huayu was used to produce the different peanut preparations. The roasting was carried out at 170°C for 20 min, frying was done in pure vegetable oil (Jinlongyu, Shenzhen, China) for 5 min, and boiled peanut was cooked for 20 min in water (100°C). Huayu in a raw form was used as control. The proteins were extracted with various buffers as shown in Table I, after the different treatments, and the extraction methods were the same for the three peanut varieties.

Table I. List of tested extraction buffers.

No.	Buffer	Concentration and composition	pH
1	Citrate buffer	0.1 M sodium phosphate and 0.05 M citric acid	4.5
2	Acetic acid buffer	0.05 M acetic acid and 0.05 M sodium acetate	5.0
3	PBS pH 6.7	0.025 M sodium phosphate	6.7
4	PBS pH 7.6	0.02 M sodium phosphate	7.6
5	PBS pH 7.6 (NaCl)	0.02 M sodium phosphate and 1M sodium chloride	7.6
6	Urea	6 M Urea and 0.02 M sodium phosphate	8.2
7	TBS	0.01 M Tris and 0.01 M HCl	8.5
8	Borate buffer (0.025 M)	0.025 M borate and 0.025 M sodium hydroxide	9.6
9	Borate buffer (0.05 M)	0.05 M borate and 0.05 M sodium hydroxide	9.6
10	Sodium carbonate buffer	0.05 M sodium carbonate and 0.05 M sodium hydrogen carbonate	10.8

SDS-PAGE and SDS-PAGE densitometry

SDS-PAGE was performed essentially according to Laemmli (1970) with AE6400 Dual Mini Slab electrophoresis apparatus (ATTO Corporation, Japan) with acrylamide gels (90 mm × 73 mm × 1 mm), and 12.5% separating gel and 4.5% stacking gel. Pre-stained molecular weight markers (Tiangen, Beijing, China) with molecular weights of 14.4, 20.0, 28.5, 35.0, 45.0, 66.2, and 94.0 kDa were used as reference. Peanut protein extracts were mixed in a 1:1 ratio with 50 mM TRIS buffer (pH 6.8) containing 1% SDS, 1% β-mercaptoethanol, and 20% (v/v) glycerin, and 0.1% bromophenol blue as tracking dye solution. Samples were boiled for 5 min before they were loaded on the gel, 25 µl (1 ug/ul) per well. Gels were stained with Coomassie brilliant blue R-250 dissolved in destaining solution [10% acetic acid (v/v), 5% methanol (v/v) in water]. After destaining, gels were scanned with gel image analysis system (Gelpro, America). The relative contents of stained peanut protein bands were quantified by densitometry (Koppelman et al. 2001).

Immunoblotting

Immunoblotting was carried out with patient sera from 10 eczema patients with positive serum IgE to peanut. Peanut specific IgE was detected by an ELISA method (Iman et al. 1990). All patients were from the Department of Dermatology, Peking University Third Hospital.

After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (pore size 0.45 µm, Sartorius, Germany) by blotting for 4.5 h in 25 mM tris buffer containing 192 mM glycine, 20% methanol (pH 8.3) using a transfer unit (ATTO Corporation, Japan) generally as described by Towbin et al. (1979). The nitrocellulose membrane was cut into halves, one was visualized by cyanamid (0.2% in acetic acid) staining, and the other was used for immunochemical detection. The membrane was washed with wash buffer (10 mM Tris, pH 7.4, containing 0.8% NaCl and 0.05% (w/v) Tween 20) thoroughly 4 times, 15 min per wash and was, then blocked with 1% bovine serum albumin, BSA (Sigma, USA) in wash buffer for 1 h. After washing with distilled water for 5 min, patient serum was diluted in 1:6 with blocking buffer (1% bovine serum albumin in wash buffer) and applied on the membrane overnight. After washing with wash buffer for 3 times, 10 min per wash, bound IgE was detected using a commercial anti-human IgE conjugated to peroxidase (Zymed, USA, lot:51101765) in a dilution of 1:800 (v/v) with blocking buffer, and

subsequently stained for peroxidase activity with 4-chloro-1-naphtol staining. All work above was carried out at 37°C. The blots were dried and scanned with a gel image analysis system (Gelpro, USA).

Immunoblot inhibition

To exclude nonspecific IgE binding to protein extracts of raw peanuts, immunoinhibition was carried out. 300 μ l of pooled positive sera and negative sera from patients, were diluted with 1% BSA in wash buffer, and were preincubated with 25 μ g peanut protein denaturated by heat treatment. Afterwards, immunoblotting was performed as described above.

N-terminal amino acid sequencing

After SDS-PAGE electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) (Immobilon-P, Millipore Corp. Bedford, MA, USA) membranes using an electro transfer buffer (10 mM (3-cyclohexylamino)-1-propane sulphonic acid (CAPS), pH 11.0, 10% (v/v) methanol) for 75 min at 0.8 mA/cm². Prior to use, membranes were rinsed in methanol (a few seconds) and electrotransfer buffer (15 min). Proteins were visualized with 0.1% (w/v) Coomassie blue R-250 in methanol/20% acetic acid (50/50, v/v) staining. After staining, the PVDF membranes were destained with 50% methanol, then washed with double-distilled water (5 min) and air-dried (Matsudaira 1987). Microsequencing of the N-terminal amino acids of major allergens was performed by Edman degradation with PROCISE 491 protein sequencer (Applied Biosystems Procise, Germany).

ELISA

The IgE binding ability of peanut proteins from different processed peanuts was determined by IgE-binding experiment as reported by Koppelman *et al.* (1999). A total of 96 well plates were coated with 15 μ g/ml peanut extracts using TBS as coating buffer. Subsequently, wells were blocked with 1% (w/v) BSA (in TBS containing 0.1% (w/v) Tween 20, blocking buffer) to diminish the nonspecific binding. Patient sera were diluted in 1:6 with blocking buffer and serum dilutions were incubated on the coated wells for 2 h at 37°C. Bound IgE was detected using an anti-human IgE antibody conjugated to peroxidase (Zymed, USA, lot: 51101765) in a dilution of 1:800 as 490 nm. Between each step, plates were washed five times with TBS containing 0.1% Tween 20. Non-specific binding was studied using serum from several non-allergic individuals as well as from an allergic (but not peanut allergic) individual. Both appeared to be negligible.

Results

SDS-PAGE for three peanut varieties

A number of bands are visible after electrophoresis on SDS-PAGE. Their molecular weights are mainly between 14 and 66 kD as shown in Figure 1A. Band patterns were similar to a great extent in all three peanut varieties except that one band (line 1) at

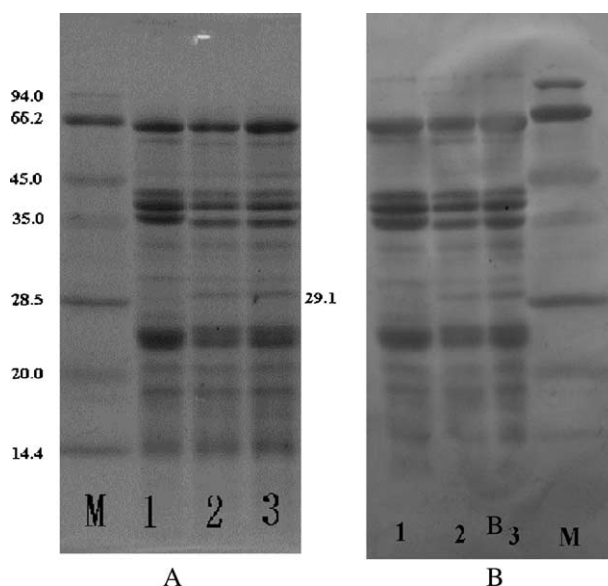


Figure 1. SDS-PAGE stained with Coomassie blue (A) and the protein profile after transfer to a nitrocellulose membrane (B), stained with cyanamid. M are markers at 94.0, 66.2, 45.0, 35.0, 28.5, 20.0 and 14.4 kD. Lane 1, 2 and 3 represent the three peanut varieties of Baisha, Luhua and Huayu, respectively.

approximately 29.1 kD was substituted by one band at approximately 23.6 kD for Baisha. This minor discrepancy may be due to the difference in peanut varieties.

Immunoblotting for three peanut varieties

All bands on SDS-PAGE were transferred to the nitrocellulose membrane as shown in Figure 1B. Immunoblotting results with patient sera were shown in Figure 2A and 2B. Five bands at 63, 58, 41, 37.5 and 23.6 kD showed specific binding to anti-human IgE in Figure 2A, and only two bands at 63 and 58 kD in Figure 2B. Immuno-inhibition results are shown in Figure 3 with the peanut variety Huayu, and the results showed that the visibility of the five bands on nitrocellulose membrane decreased after pre-incubation. Among the positive bands in immunoblotting, the bands at 35, 29.1 and 14.4 kD exhibited non-specific binding to anti-human IgE (Figure 2C). The IgE-binding activity of peanut proteins are shown in Table II. Two bands at 63 and 58 kD were recognized by IgE in 100% of peanut-sensitized patient, and thus considered as dominant peanut allergens among the serum tested. The sub-units at 41, 37.5 and 23.6 kD were recognized by IgE in 80% peanut allergenic patients, and also considered as major allergens. On the other hand, the bands at 30.3 and 18.7 kD also contribute to the allergenicity of peanut proteins. The 47, 32.8 and 20.8 kD proteins are considered as minor peanut allergens.

N-terminal amino acid sequencing

N-terminal amino acid residues of the five major peanut allergens were analyzed by microsequence as shown in Table II. N-terminal sequences of the 63 kD band were matching the amino acids of Ara h 1 to 100%, and of the 58 kD band were matching

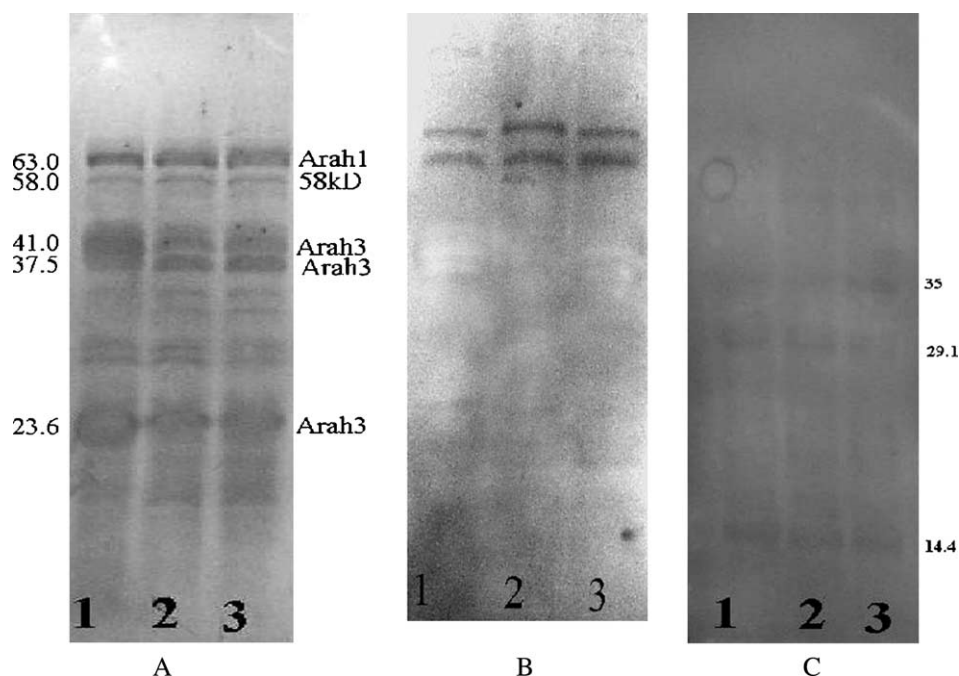


Figure 2. Immunoblotting of raw peanuts with positive and negative patient sera. Lane 1, 2 and 3 represent the three peanut varieties of Baisha, Luhua and Huayu, respectively. In (A) and (B), two different peanut allergic patient sera were used for blotting (the other immunoblotting of raw peanut with positive sera from 8 patients are the same to [a], and thus only 2 figures are shown) and (C) represents immunoblotting of raw peanut with negative sera.

the amino acids 150–159 of Ara h 1 to 90%. The 41 and 37.5 kD proteins have N-terminals identical to Ara h 3 to 100%, and the band at 23.6 kD is identical to Ara h 3 to 90%.

Protein patterns on SDS-PAGE with various buffers

Protein patterns were shown for the four different methods of peanut preparations in various extraction buffers (Figure 4). With citrate buffer (pH 4.5) and acetic acid buffer (pH 5.2), very few of the major allergens were extracted. With other buffers at pH 6–11, a number of bands are visible on SDS-PAGE, mainly ranging between 14 and 66 k D. Band patterns were similar to a great extent in all samples except that the 58 kD band was only present in raw peanuts in PBS pH 7.6 buffer and PBS pH 7.6 (NaCl). In addition, the 63 kD band was clearly reduced in fried peanut extracted with PBS, pH 7.6 (Figure 4, lane F and Figure 5), and it was almost not visible in boiled peanut with PBS pH 7.6 (NaCl) (Figure 4, lane B and Figure 5). In contrast, the 41, 37.5 and 23.6 kD fragments seem to be similar in all 4 methods of preparations with buffers at pH 6.7–10.8.

The effect of buffer composition on peanut allergens extraction efficiency

A quantitative determination of peanut allergens extraction efficiency was done by SDS-PAGE densitometry (Figure 5). Extraction with PBS buffer at pH 7.6, the

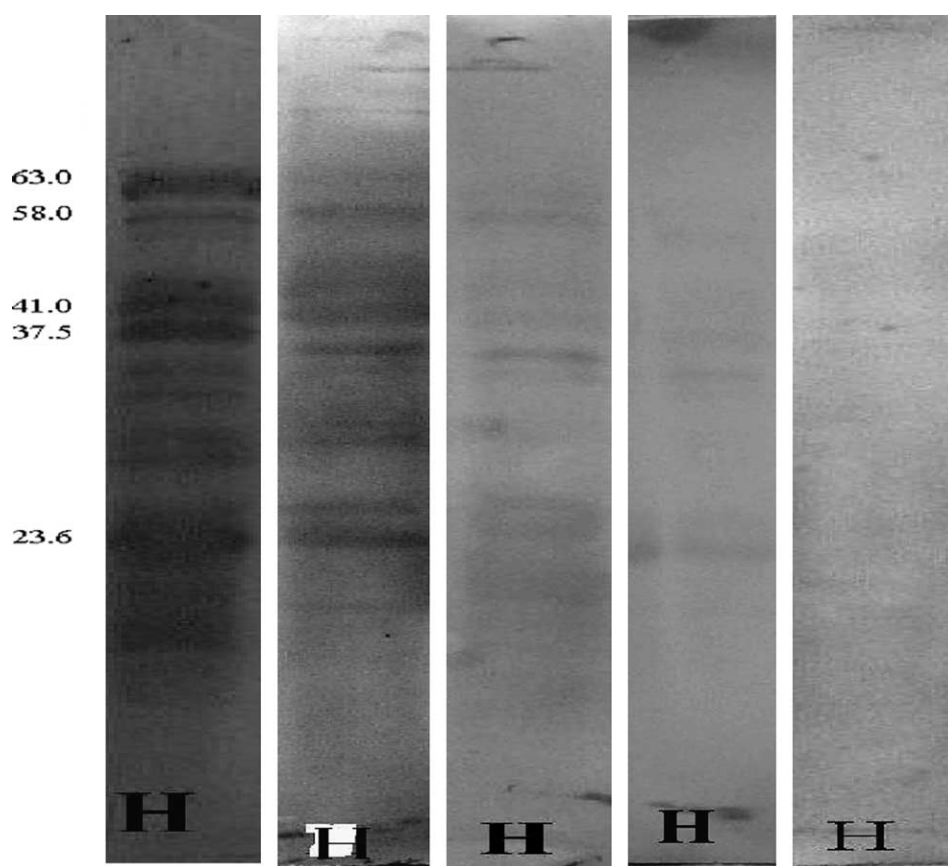


Figure 3. Immunoinhibition of peanut protein with pooled positive patient sera. (a) Represents immunoinhibition of peanut protein with pooled positive patient sera; (b) represents immunoinhibition of peanut protein with pooled positive patient sera diluted in 1/1; (c) represents immunoinhibition of peanut protein with pooled positive patient sera diluted in 1/3; (d) represents immunoinhibition of peanut protein with pooled positive patient sera diluted in 1/5; (e) represents immunoinhibition of peanut protein with pooled positive patient sera diluted in 1/7; H represents Huayu.

amount of 63 kD protein in fried peanut was less than of roasted peanut. Also in PBS pH 7.6 (NaCl), there was almost the same trend, but the total extraction efficiency was higher. However, with other buffers, the content of the 63 kD protein was almost higher in fried peanuts compared to roasted peanut. In boiled peanut the 63 kD protein was higher than in roasted peanuts except at pH 6.7 and 10.8. Best yields of the 63 kD protein was obtained with PBS at pH 6.7 for all peanut preparations. There was a significant difference in the relative content of 63 kD for peanut preparations in all buffers ($p < 0.05$). No significant difference was observed in the relative content of the 41, 37.5 and 23.6 kD proteins in the different peanut preparations.

The effect of frying, boiling and roasting on the allergenicity of peanuts

As shown in Figure 6 (A1, B1), four major peanut allergens can be observed with immunoblotting. Control tests were carried out with pooled negative sera (Figure 6, A2

Table II. Identification of peanut allergens based on N-terminal amino acid sequence and IgE-binding activity of peanut proteins.

Proteins	The N-terminal sequence	Equivalence to sequence of known peanut allergen	Alignment (amino acid residue)	Known peanut allergens	Identity	References	IgE-binding activity (number of patients with reactions/ total patients number)
63 kD	R S P P	R S P P R S P P	85–88 1–4	Ara h 1	4/4	Burks et al. (1995), Buschmann et al. (1996)	10/10
58 kD	E G R E G E Q E E G	E G R E G E Q E W G	150–159	Ara h 1	9/10	Burks et al. (1995)	10/10
41 kD	I S F R Q Q P E E N	I S F R Q Q P E E N	1–10	Ara h 3	10/10	Rabjohn et al. (1999)	8/10
37.5 kD	I S F R Q Q P E E N	I S F R Q Q P E E N	1–10	Ara h 3	10/10	Rabjohn et al. (1999)	8/10
23.6 kD	G I E E T I C I A S	G I E E I I C I A S	326–335	Ara h 3	9/10	Rabjohn et al. (1999)	8/10

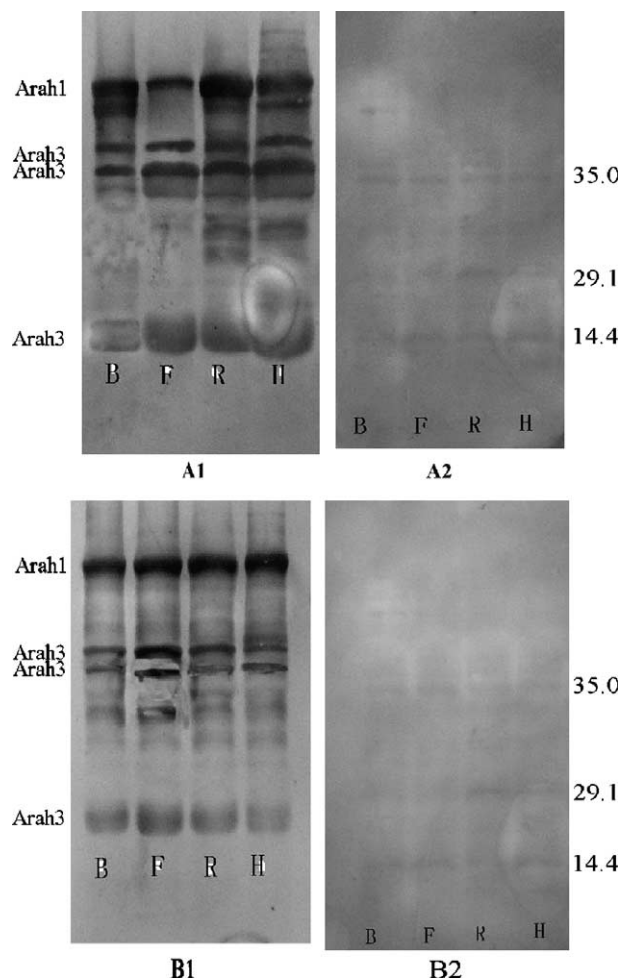


Figure 6. Immunoblotting of cooked peanuts with pooled positive and negative patient sera. Lanes H–B represent raw (Huayu), roasted, fried and boiled peanuts in order. (A1) Represents immunoblotting of cooked peanuts extracted with Urea buffer with pooled positive patient sera; (A2) represents immunoblotting of cooked peanuts extracted with Urea buffer with pooled negative sera; (B1) represents immunoblotting of cooked peanuts extracted with PBS pH 7.6 with pooled positive patient sera; (B2) represents immunoblotting of cooked peanuts extracted with PBS pH 7.6 with pooled negative sera.

and B2). Slight non-specific binding to bands at 35, 29.1 and 14.4 kD were seen. The IgE binding ability of peanut proteins from different processed peanuts was determined by ELISA, data as followed: the OD values of raw, roasted, fried and boiled peanuts ($\pm$ SD) were 0.792 ± 0.03 , 0.746 ± 0.03 , 0.814 ± 0.03 , and 0.773 ± 0.03 , respectively ($p > 0.05$) with Urea buffer extraction, while the OD value were 0.787 ± 0.01 , 0.828 ± 0.02 , 0.738 ± 0.01 , 0.725 ± 0.02 , respectively, ($p < 0.05$) with PBS pH 7.6 extraction.

Discussion

Peanut allergy in China has increased to 4% in food allergy patients (Hill et al. 1997; Li & Zhang 2001) and it is of interest to study the major peanut allergens in China.

To rule out the non-specific binding in immunoblots, an immunoinhibition assay was performed with pooled positive sera and pooled negative sera. Immunoinhibition test showed that the IgE binding to the five major bands on nitrocellulose membrane decreased after pre-incubation indicating that binding was specific for peanut IgE in the peanut allergic sera.

Based on their N-terminal sequences, the band at 63 kD is identical to Ara h 1 (Burks et al. 1995; Buschmann et al. 1996; De Jong et al. 1998). The 41 and 37.5 kD proteins have N-terminals identical to the 42 kD peanut allergen found by Rabjohn et al. (1999). They are therefore likely both sub-units of Ara h 3. In addition, the N-terminal amino acid residues of the band at 23.6 kD is identical to the 25 kD peanut allergen to 90% found by Rabjohn et al. (1999). Furthermore, N-terminal sequences of the 58 kD band were matching the amino acids 150–159 of Ara h 1 to 90% (Burks et al. 1997) suggesting that this is an isoform of Ara h 1.

Ara h 1 was one of the dominant allergen in peanut and supported the findings of (Burks et al. 1991; De Jong et al. 1998). Burks et al. (1995) have cloned the Ara h 1 gene and based on the cDNA, they have predicted the entire N-terminal amino acid sequence. Our data revealed that the N-terminal amino acid residues of Ara h 1 were identical to the sequence of Burks et al. at position 85–88. In addition, the 58 kD protein was the isoform or fragment of Ara h 1 since the N-terminal amino acid residues of the 58 kD protein were 90% identical to the sequences of Burks et al. at position 150–159. The discrepancy may be due to differences in protease processing or alternative splicing. The cleavage sites of Ara h 1 and the 58 kD protein were an arginine and glutamic acid residue respectively (Burks et al. 1995). This study implies that Ara h 1 present in peanuts is a truncated form of the cDNA derived gene which authenticates the hypothesis of De Jong et al. (1998).

The major allergens at 41, 37.5 and 23.6 kD belongs to Ara h 3 (Eigenmann et al. 1996). Ara h 3 belongs to the glycinin storage protein family (Burks et al. 1991; Eigenmann et al. 1996; Rabjohn et al. 1999), and the presence of acidic and basic subunits can be explained according to homology with soy glycinin, the best characterized protein of the glycinin family. The cysteines responsible for the disulfide bond covalently link the acidic sub-unit (res 1–325) and the basic sub-unit (res 326–510) are cysteine 88 (acidic sub-unit) and cysteine 332 (basic sub-unit) (Koppelman et al. 2003). Alignment shows that the 23.6 kD protein starts at amino acid 326, which is the acidic/basic sub-unit site of cleavage, indicating incomplete processing of the Ara h 3 preprotein.

Although several peanut allergens were detected in raw peanuts, different ways of cooking might affect the allergenicity of peanuts differently. Frying and boiling are common cooking methods in China, while roasted peanuts are more commonly consumed in the US. The effect of buffer composition on peanut allergens extraction efficiency was evaluated (Figures 4 and 5). It can not be excluded that different buffers lead to different extraction efficiency for protein fragments. Therefore it would be desirable to establish a standard buffer system for peanut protein extraction. Through extraction with PBS buffer at pH 7.6, the amount of Ara h 1 in fried peanut is less than in roasted peanut (Figure 4), which is in line with the study of Beyer et al. (2001) showing that Ara h 1 in general was markedly reduced in fried peanut compared to in the roasted form. However, with other buffers (pH 8.2–10.8), the contents of Ara h 1 were almost higher in fried peanut compared to in roasted peanut. In relation to raw

peanuts, the extraction efficiency decreased by processing supporting the study of Poms et al. (2004).

The ELISA showed that frying, boiling and roasting had no effect on the allergenicity of peanut compared to raw peanut extracted with Urea buffer. Compared with the reduced quantities of Ara h 1 with PBS at pH 7.6, the IgE-binding ability to Ara h 1 was also significantly reduced by frying compared with roasting ($p < 0.05$). This result was consistent with the result of Beyer et al. (2001), but different from the result of Maleki et al. (2000). The last authors showed that roasting may enhance the allergenic properties of peanuts compared to those in the raw form. The discrepancy between our study and the previous study may be due to the different genetic background of the allergic population.

In conclusion, Ara h 1 and the 58 kD protein were dominant allergens and a series of Ara h 3 were major allergens in China. Different buffers can lead to different efficiency of peanut allergen extraction for raw, roasted, fried and boiled peanuts. There were significant differences in the relative content of Ara h 1 for peanut preparations in the tested buffers ($p < 0.05$), but no significant differences were observed in the relative contents of Ara h 3 fragments. Cooking methods may not explain why the prevalence of peanut allergy is much lower in China compared to the US and some countries in Europe.

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