

Original article

Quantification of major peanut allergens Ara h 1 and Ara h 2 in the peanut varieties Runner, Spanish, Virginia, and Valencia, bred in different parts of the world

Background: The serology of peanut allergy seems to be different in various parts of the world. We analyzed the composition of 13 samples of three varieties of peanut in order to compare their allergenic nature.

Methods: Peanut cultivars that are commonly processed in the West were analyzed for protein content, protein composition, and Ara h 1 and Ara h 2 content by biochemical methods. IgE-binding properties were analyzed by ELISA using serum from patients with documented peanut allergy.

Results: Total protein contents were comparable for all tested samples (24–29%), and proteins were extractable to the same extent. SDS–PAGE patterns differed slightly, but all major bands were visible in all samples (molecular masses of approximately 14–100 kDa under reducing conditions). Ara h 1 and Ara h 2 were quantified by SDS–PAGE densitometry and were expressed as percentage of the total protein content. Ara h 1 was in the range 12–16%, whereas Ara h 2 was 5.9–9.3%. In view of the analytic uncertainty of this determination, the content of both Ara h 1 and Ara h 2 was not significantly different between the tested samples. In an IgE-binding inhibition ELISA, the affinities of the peanut proteins for peanut-specific IgE were measured. Minor differences were observed between the tested samples, with the most potent IgE-binding sample having a two times higher ability to bind IgE than the weakest IgE-binding sample.

Conclusions: The results suggest that peanuts of different varieties and from different parts of the world contain similar proteins, including Ara h 1 and Ara h 2. Consequently, the IgE-binding properties are similar to a great extent. This indicates that differences in the serology of peanut allergy may not originate from differences in the allergen composition of the peanut.

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The nature of the allergenic compounds in peanuts has been studied extensively in recent years (1–3), and two major peanut allergens, Ara h 1 (4, 5) and Ara h 2 (5, 6), have been identified. Purified Ara h 1 has been the subject of some biochemical and immunologic studies, and was found to be a 63-kDa glycoprotein (4, 7) with distinct, heat-stable IgE-binding sites (8), both on the protein part (9–11) and on the carbohydrate moiety of the molecule (12). Ara h 2 was purified from peanuts, and it migrated as a doublet at 17–20 kDa on reducing SDS–PAGE (5, 6). The genes encoding Ara h 1 (13, 14) and Ara h 2 (15) have been cloned, and the gene products resembled similar biochemical and immunological properties, although the post-translational processing of the recombinant allergens may be some-

what different from the peanut-derived allergens (5). Sequence analysis of Ara h 1 showed a significant homology with the vicilin seed storage protein family (11), and Ara h 2 seemed to belong to the conglutin family (15).

In contrast to the biochemical and immunologic knowledge of individual peanut allergens, there is limited information on the possible differences in allergen composition among peanut species or cultivars. One study presented IgE-binding properties of crude extracts of two varieties bred in the USA (Virginia and Runner), concluding that the proteinaceous parts were responsible for IgE binding (16), but cultivars from other parts of the world were not tested. In those days, major allergens had not been identified; consequently, no information on the allergen composition in the two

cultivars was given. In population studies, Ara h 1 and Ara h 2 were identified as major allergens. A striking observation was that Ara h 1 was recognized by over 95% of the patients from a North American population (4) whereas the same allergen was recognized by only 35% (5), 65% (17), or 70% (18) of the patients of three European populations. These differences in relative recognition were not found for Ara h 2.

This study aimed to investigate whether possible differences in allergen composition of the commonly consumed peanut varieties (Fig. 1 [19]) may explain the differences in the serology of peanut allergy.

Material and methods

Peanut samples

Peanuts (*Arachis hypogaea*) from various varieties were generously provided by Imko Nut Products (Doetinchem, The Netherlands) and were stored at 10°C until use. Table 1 summarizes the varieties, origin, and size (number of peanut kernels per 100 g).

Protein extraction methods

Peanut protein extracts were made by mixing 1 g ground peanut with 20 ml 20 mM Tris (pH 8.2). After 2 h of stirring at room temperature, the aqueous fraction was collected by centrifugation (3000 g, at room temperature for 5 min). The aqueous phase was subsequently centrifuged (10 000 g at room temperature for 15 min) to remove residual traces of fat and insoluble particles. Extracts were stored at -20°C until use. The protein content of both ground peanut and peanut extracts was determined by the Kjeldahl method.

Averages of three independent experiments are shown. To illustrate the analytic precision of this method, the values of sample 01 ($\pm$ standard deviation) were $24\% \pm 1\%$ for total protein and $20\% \pm 3\%$ for extractable protein. All samples (within one type of analysis) resulted in similar standard deviations; for the sake of clarity, standard deviations are omitted in Table 2.

SDS-PAGE

SDS-PAGE was performed essentially according to Laemmli (20) with a BioRad Mini Protean II system (BioRad, Hercules, CA, USA) with 15% acrylamide gels (15 $\times$ 10 cm). Prestained molecular weight markers with molecular weights of 14.3, 21.5, 30, 46, 66, 97.4, and 220 kDa were used as reference. Soluble samples (i.e., peanut protein

Table 2. Protein content, Ara h 1 and Ara h 2 content, and IgE-binding properties

Sample code	Total protein (%)	Extractable protein (%)	Ara h 1 (% from total protein)	Ara h 2 (% from total protein)	IgE IC ₅₀ (ng/ml)
01	24	20	13	6.2	121
02	24	22	14	6.3	117
03	28	26	13	6.6	125
04	26	22	16	7.7	98
05	28	26	15	6.6	98
06	29	18	16	8.3	73
07	26	24	16	6.1	121
08	28	20	15	6.7	94
09	28	26	14	5.9	126
10	27	25	12	9.2	103
11	29	23	16	8.0	100
12	26	18	14	8.0	93
13	29	24	14	9.3	88

extracts) were mixed in a 1 to 5 ratio with 63 mM TRIS buffer (pH 6.8) containing 1% dithiotreitol (DTT), 2% SDS, 0.01% bromophenol blue, and 40% (v/v) glycerol, and were subsequently boiled for 5 min. Solid samples (peanut meals, 10 mg) were extracted directly with 1 ml 63 mM TRIS buffer (pH 6.8) containing 1% dithiotreitol (DTT), 2% SDS, 0.01% bromophenol blue, and 20% (v/v) glycerol. Samples of 10 μ l were loaded on the gel. Gels were stained with Coomassie brilliant blue R-250 dissolved in destaining solution (10% HAc (v/v), 5% methanol (v/v) in water). After destaining, gels were scanned with an Image-Master DTS (Pharmacia, Uppsala, Sweden). Purified peanut-derived Ara h 1 and Ara h 2 were used as references for band assignment (8). Bands were quantified by densitometry, and Ara h 1 and Ara h 2 were expressed as percentage of total protein. Averages of three independent experiments are shown. To illustrate the analytic precision of this method, the values of sample 01 ($\pm$ standard deviation) were $13\% \pm 2\%$ for Ara h 1 and $6.2\% \pm 1.3\%$ for Ara h 2. All samples resulted in similar standard deviations, and for the sake of clarity, standard deviations are omitted in Table 2.

Patient sera

A pool of serum from eight adult patients with documented peanut allergy from a Dutch population was used to study the interaction of peanut proteins with IgE. Each of these patients had a positive skin prick test to peanuts and a convincing history of peanut anaphylaxis. The presence of peanut-specific IgE was established with Cap FEIA (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden, ranging from 39 to >100 kU/l) and in immunoblotting experiments (not shown). Both a nonallergic subject and an allergic, but not peanut-allergic, subject were used as controls. Venous blood was drawn from the individuals and allowed to clot. Serum was collected by centrifugation and stored in aliquots at -20°C until use. All studies were approved by the Medical and Ethical Committee of the University Medical Center of Utrecht (Utrecht, the Netherlands), and informed consent was obtained from each patient.

IgE-binding studies

A quantitative determination of the ability of peanut proteins to bind to IgE was made by IgE-binding experiments, generally according to Burks et al. (21). Dilutions of peanut protein extracts (final concentrations 0.003–100 μ g/ml) were incubated with a 1 to 30 dilution of pooled patient serum in phosphate-buffered saline (PBS) containing 1% BSA and 0.1% Tween 20. In this fluid phase, peanut proteins were allowed to bind to IgE for 1 h at room temperature

Table 1. Description of peanut samples used

Sample code	Varieties	Origin	Size
01	Runner	USA	60/70
02	Runner	USA	40/50
03	Runner	Argentina	40/50
04	Runner	Argentina	38/42
05	Virginia	USA	28/32
06	Virginia	USA	21/26
07	Virginia	China	35/45
08	Virginia	China	25/29
09	Spanish	USA	60/70
10	Spanish	South Africa	60/70
11	Spanish (hybrid)	Nicaragua	60/70
12	Valencia	China	60/70
13	Valencia	USA	60/70

Taxonomic classification of the peanut

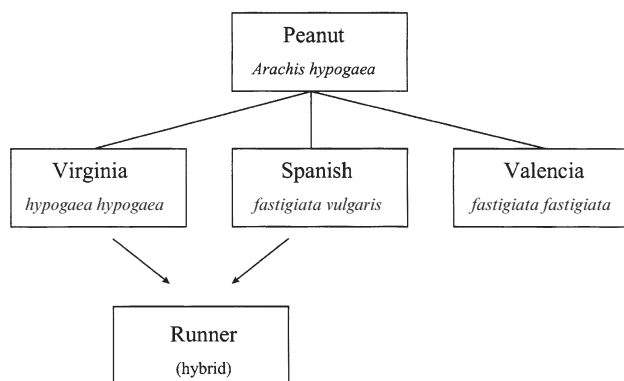


Figure 1. Taxonomic classification of peanut according to Krapovickas (19). Peanut species (*A. hypogaea*) comprises two subspecies (*hypogaea* and *fastigiata*), latter divided into two varieties (*fastigiata vulgaris* and *fastigiata fastigiata*). Runner type is hybrid of *fastigiata* and *hypogaea* subspecies, and since this type has long been used, Runner is often regarded as separate variety. For clarity, Virginia, Spanish, and Valencia, as well as Runner, will be regarded as individual varieties.

under gentle shaking. In order to determine the unbound IgE fraction, the incubation mixtures were transferred to the 96-well plates pretreated as follows: 96-well plates were coated with 10 µg/ml peanut protein (Runner, USA, sample code 01) in PBS and subsequently blocked with BSA (1% in PBS containing 0.1% Tween 20) to diminish the nonspecific binding. IgE bound to the coated wells was detected with an antihuman IgE antibody conjugated to horseradish peroxidase (product code MELIE 1–2, DPC, Los Angeles, CA, USA, diluted five times in PBS containing 0.1% Tween 20). Between each step, the plates were washed five times with PBS containing 0.1% Tween 20. The inhibition of IgE binding as a function of the amount of peanut protein present in the preincubation sample reflects the ability to bind to IgE. The concentrations needed for half-maximal inhibition were calculated with a semilogarithmic equation and were used to compare this ability in a quantitative manner. Averages of three independent experiments are shown. To illustrate the analytic precision of this method, the value of sample 01 ($\pm$ standard deviation) was 121 ± 12 ng/ml. All samples resulted in similar standard deviations; for the sake of clarity, standard deviations are omitted in Table 2.

Results

Since protein is recognized as the allergenic substance in peanut, we first analyzed the protein content of the different peanuts. Total protein content and extractable protein were measured by the Kjeldahl method (Table 2). The total protein content varied between 24% and 29%, and the extractable protein varied between 18% and 26% for the tested samples. The standard deviation of these determinations was 2%. Electrophoresis was used to compare the protein composition of the different peanuts. Fig. 2A shows the pattern of sample 01 (USA Runner [60/70]), for example. To identify the individual bands in this sample, purified Ara h 1 (approximately 63 kDa) and Ara h 2 (doublet at approximately 17–20 kDa) are used as

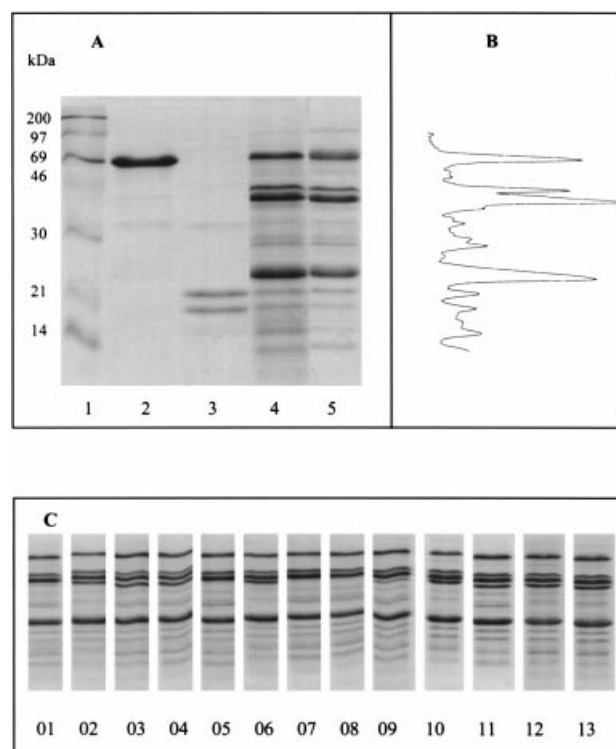


Figure 2. Electrophoresis of peanuts. Panel A: lane 1) marker proteins (indicated at left); lane 2) purified Ara h 1; lane 3) purified Ara h 2; lane 4) meal from sample 01 (Runner, USA [60/70]); lane 5) Tris (pH 8.2) extract from sample 01 (Runner, USA [60/70]). Panel B: densitometry of sample 01 (Runner, USA [60/70]). Panel C: electrophoretic patterns of all tested samples. Lane numbers refer to sample codes.

markers. The relative amounts of Ara h 1 and Ara h 2 in the USA Runner (60/70) were calculated by densitometry (Fig. 2B). Fig. 2C shows the SDS-PAGE patterns of the other tested peanuts. Band patterns were similar to a great extent. However, one band at approximately 40 kDa was absent in four of the 13 tested samples. In all samples, Ara h 1 and Ara h 2 were present. Table 2 (columns 4 and 5) depicts the relative content of these major allergens as determined by densitometry (see also Fig. 2B). The amounts of Ara h 1 varied between 12% and 16%, and those of Ara h 2 between 5.9% and 9.3%. The standard deviations of these determinations were 2% and 1.3%, respectively, indicating that the levels of Ara h 1 and Ara h 2 were not significantly different between the tested samples.

The total peanut protein content and the Ara h 1 and Ara h 2 content indicated minor differences between the tested peanuts. For assessment of the allergenicity of the different peanuts, the IgE-binding properties of the various peanuts were tested in an IgE-binding inhibition assay. Fig. 3 shows an example for sample 01 (USA Runner [60/70]). With increasing doses of peanut protein in the soluble phase, the binding of IgE to immobilized peanut protein decreased. The concentration of peanut protein to reach 50% inhibition of IgE

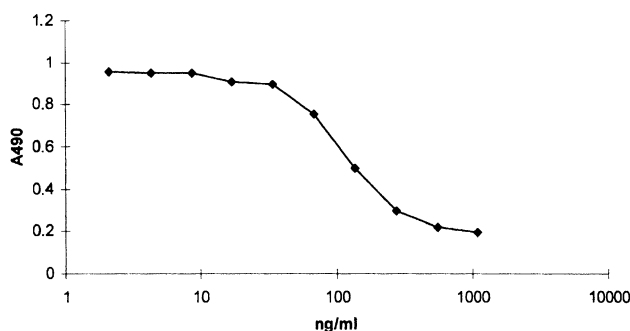


Figure 3. Inhibition of IgE binding. Dilutions of sample 01 (Runner, USA [60/70]) were incubated with serum from peanut-allergic patients. Unbound IgE was captured on peanut protein-coated plates and stained with peroxidase-conjugated antibody directed against human IgE.

binding (IC_{50}) was used to compare the abilities of the different peanuts to bind to IgE (Table 2, column 6). As expected from the similarity of previously analyzed parameters, the IC_{50} values varied only slightly, between 126 (lower binding ability) and 73 ng/ml (higher binding ability).

Discussion

In recent years, peanut allergens have been identified and characterized to a large extent. In particular, the allergens Ara h 1 and Ara h 2 have been a focus of attention, and their biochemical and immunochemical properties have been documented. However, in populations of patients with peanut allergy in the USA, Ara h 1 and Ara h 2 are considered to be major allergens (4, 6) (recognized by more than 90% of the peanut-allergic patients), whereas in three different populations from Europe Ara h 1 was recognized by lower percentages of patients (35% [5], 65% [17], and 70% [18]). The reason for this discrepancy is not known.

The differences may be due to genetic, dietary, or other population differences, or to variations in the peanuts that are consumed. We hypothesized that peanuts of different varieties, and peanuts grown in different parts of the world might consist of different allergens, leading to different sensitization and serology.

We analyzed the three peanut varieties (*A. hypogaea*) that are commonly used for human consumption (Valencia, Virginia, and Spanish) and selected harvests from different parts of the world. Although an extensive number of species, subspecies, and varieties of the peanut were identified at the beginning and middle of the 20th century, only cultivars of Virginia (*A. hypogaea* subspecies *hypogaea*), Spanish, and Valencia (both *A. hypogaea*, subspecies *fastigiata*) are now used for breeding. The Runner peanut is a hybrid of the Virginia and Spanish subspecies, and since this cultivar has been used for a long time, it is sometimes regarded as a variety (Fig. 1). Whereas peanut consumption in the

USA is mainly domestic, peanuts in Europe are imported from various parts of the world. The USA (mainly Georgia), South America (mainly Argentina and Brazil), the Far East (China and India), and Africa (South Africa) are the most important exporters to the European market. Peanut production in Europe counts for only 1–2% of the total European peanut consumption (22). In sum, the peanut varieties selected and breeding locations reflect the main differences between peanut consumption in the USA and Europe. In earlier comparative studies, Basha et al. (23, 24) and Cherry et al. (25) analyzed protein content in six peanut samples, all of the Runner type, and all bred in the USA (Georgia). Analyses comprised total protein content, free amino acids, carbohydrates, and protein pattern by disk electrophoresis. In those days, peanut allergy was less recognized, and peanut allergens had not been identified. Therefore, there is no detailed information from those studies on allergen content. In our study, we first analyzed the total protein content of these peanuts since the proteinaceous part of the peanut has been demonstrated to be the allergenic compound. Total protein contents varied between 24% and 29%, reflecting the normal range of total protein content of 25–29% (26) that is used for trading purposes. There was no correlation between the total protein concentration and varieties or breeding location. The extractable protein levels were somewhat lower than the total protein content. We have used an extraction buffer with a pH of 8.2, which is optimal for peanut protein extraction (23, 26). With lower pHs, a fractionation of peanut proteins would have occurred since most peanut proteins have isoelectric points between 3 and 6.5 (26). In fact, to enrich a peanut extract in Ara h 1, an extraction buffer with pH 7.2 is used. This results in an extract with an Ara h 1 concentration of approximately 50% compared to total protein (5, 27). The fact that the extractable protein levels were lower than the total protein contents may be explained by our mild grounding and extraction method to avoid protein aggregation. SDS-PAGE patterns from extracted protein and from peanut meal indicated only minor quantitative differences in band intensities, whereas the band patterns did not differ in composition (Fig. 1A, lanes 4 and 5). Altogether, the composition of the peanut protein extracts reflects to a great extent the protein composition of the peanut meals.

Using purified Ara h 1 and Ara h 2 as marker proteins on SDS-PAGE, we have identified these allergens in the band patterns of each peanut sample. With gel densitometry, the relative amounts of Ara h 1 and Ara h 2, as percentage of the total protein content, were quantified. In all cases, Ara h 1 was more abundant than Ara h 2, consistently with earlier reports (4–6, 27), and the levels varied between 12% and 16% and 5.9% and 9.3% of the total protein content, respectively. Considering the analytic uncertainty of the applied determinations,

these values were not significantly different. Since traces of peanut allergens are thought to induce sensitization (28), the differences that we observed in amounts of Ara h 1 and Ara h 2 were probably not the explanation for the differences in the serology of peanut allergy. Next to Ara h 1 and Ara h 2, a number of additional bands are visible on SDS-PAGE, mainly between 25 and 60 kDa. One band at 30 kDa was previously identified as peanut lectin, and other bands may originate from the recently identified allergens Ara h 3 (29), Ara h 4, Ara h 5, and Ara h 6 (17). Since these allergens have been studied as recombinant gene products rather than peanut-derived proteins, their molecular weights have not been studied in detail yet. Therefore, it is difficult to assign the bands other than Ara h 1, Ara h 2, and lectin.

If we consider Ara h 1 and Ara h 2 major allergens, we could speculate that the allergenicity of peanuts depends on the content of these two allergens. However, since other allergens have been identified, too, and since their IgE-binding properties have been demonstrated, their contribution to allergenicity must also be taken into account. Therefore, we used an IgE-binding test with a pool of serum from peanut-allergic patients that reacted with a number of bands other than Ara h 1 and Ara h 2

(immunoblot results not shown). Although IgE binding does not indicate that those proteins are necessarily involved in initiating the clinical signs of allergy, such an assay provides additional information on the allergenic nature of the peanut samples. As a measure of ability to bind to IgE, we have determined the concentration of peanut protein required for half-maximal IgE-binding inhibition (IC_{50}) (8). The maximal difference in ability to bind IgE that we have found for the tested peanuts was twofold. This difference is considered to be minor because differences in IgE-binding capacity that have physiologic relevance are typically 100- or 1000-fold, as in the case of the house-dust-mite allergens Der p 1 and Der f 1 (30).

In summary, we analyzed four different peanut varieties, cultured in various parts of the world, for protein content and composition. Furthermore, Ara h 1 and Ara h 2 were quantified, and IgE-binding properties were assessed. No significant differences were found in either of the parameters; therefore, we conclude that differences in serology between peanut-allergic patients from different parts of the world are not explained by the allergen composition of different peanuts.

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