Peanut defensins: Novel allergens isolated from lipophilic peanut extract

Arnd Petersen, PhD,* Skadi Kull, PhD,§ Sandra Rennert, MSc, Wolf-Meinhard Becker, PhD,§ Susanne Krause, PhD,§ Martin Ernst, PhD, Thomas Gutsmann, PhD, Johann Bauer, PhD, Buko Lindner, PhD, and Uta Jappe, MD, MS

Borstel, Freising, and Luebeck, Germany

Background: Peanut is one of the most hazardous sources of food allergens. Unknown allergens are still hidden in the complex lipophilic matrix. These allergens need to be discovered to allow estimation of the allergenic risk for patients with peanut allergy and to further improve diagnostic measures. Objective: We performed detection, isolation, and characterization of novel peanut allergens from lipophilic peanut extract.

Methods: Extraction of roasted peanuts were performed under defined extraction conditions and examined by means of 2-dimensional PAGE. Subsequently, chromatographic methods were adapted to isolate low-molecular-weight components. Proteins were studied by using SDS-PAGE and immunoblotting with sera from patients with peanut allergy. For allergen identification protein sequencing, homology search and mass spectrometry were applied. Functional characterization for allergenicity was performed by using the basophil activation assay and for antimicrobial activity by using inhibition assays of different bacteria and fungi.

Results: IgE-reactive proteins of 12, 11, and 10 kDa were first detected after chloroform/methanol extraction in the flow through of hydrophobic interaction chromatography. The proteins were able to activate basophils of patients with peanut allergy. N-terminal sequencing and homology search in the expressed sequence tag database identified the allergens as peanut defensins, which was confirmed by using mass spectrometry. On microbial cell cultures, the peanut defensins showed inhibitory effects on the mold strains of the genera Cladosporium and Alternaria but none on bacteria.

Conclusions: We identified defensins as novel peanut allergens (Ara h 12 and Ara h 13) that react in particular with IgE of patients with severe peanut allergy. Their antimicrobial activity is solely antifungal.

(J Allergy Clin Immunol 2015;136:1295-301.)

Key words: Antifungal activity, Ara h 12, Ara h 13, food allergy, lipophilic extraction, plant defensin, peanut allergy

Peanut is one of the most hazardous sources of food allergens. As little as 100 μg of peanut protein can provoke symptoms in allergic subjects. In the majority of cases, the symptoms of peanut allergy are severe; some are even fatal. One percent of the US population is affected by peanut allergy, and the prevalence is increasing, especially in US and European children. However, it is difficult to avoid peanuts because they can be present as hidden allergens in exotic meals, in candy bars, and as supplements, such as peanut oil in cosmetics.

The reason for the strong allergenicity of peanut is due to many factors, such as the molecular complexity of peanuts, the high lipid content, and some effects of food processing on peanut proteins (ie, dry roasting of peanuts or roasting them in oil for variable times and temperatures has been shown to increase allergenicity).

As of June 2012, 10 peanut allergens had been listed in the allergen database of the World Health Organization/International Union of Immunological Societies. The allergens were usually isolated by use of aqueous buffers and at neutral pH, which is similar to the preparation of routine diagnostic extracts. Becker demonstrated that the protein and allergen patterns of peanut can vary considerably as a function of the pH value of the extraction buffer. Because of the high lipid content of up to 50%, lipophilic allergens might not be included in aqueous extracts. For example, Leduc et al reported that 10 of 34 patients with sesame allergy remained unidentified because key lipophilic allergens, such as oleosins, were not included in the aqueous diagnostic extract. The lipid phase of food extracts has thus far only partially been characterized for allergens. However, peanut contains several lipophilic allergens, such as Ara h 9, a lipid transfer protein, and Ara h 10 and 11, both oleosins. Recently, Ara h 8, the Bet v 1 homologue naturally associated with lipids, was isolated efficiently by means of chloroform/methanol extraction under alkaline conditions.

Importantly, increased IgE reactivity, as well as thermal and proteolytic stability as a function of roasting conditions and/or lipid association, was observed for Ara h 8.

In the process of purifying the lipid-associated Ara h 8, additional low-molecular-weight and thus far previously unidentified IgE-reactive peanut proteins became visible. Therefore we focused on identification and allergenic and
functional characterization of these putative allergens found in lipophilic extracts. Inclusion of lipophilic allergens potentially improves the sensitivity and specificity of aqueous extract–based diagnostics. Risk assessment and management of peanut allergy can be improved by the availability of comprehensive individual sensitization patterns facilitated by the characterization of these novel allergens (ie, by expanding the repertoire of relevant allergens in component-resolved diagnostics).

METHODS
Isolation of peanut defensin
Roasted peanuts (Seeberger Riesen; Seeberger, Ulm, Germany) were peeled and ground, and lipophilic extraction was performed, as previously described. Briefly, peanut flour (1.8 wt/vol) was dispersed in 50 mmol/L sodium carbonate (pH 11) for 1 hour at room temperature, chloroform/methanol (5:4 vol/vol) was added, and the mixture was centrifuged to separate from solid material. The organic solvent was removed from the aqueous phase by means of rotary evaporation, and the extract was dialyzed with Milli-Q water (Millipore, Temecula, Calif) overnight at room temperature. After extraction, chromatographic separation was performed on an ÄKTAprime device (GE Healthcare, Freiburg, Germany). Hydrophobic interaction chromatography (Phenyl Sepharose High Performance, GE Healthcare) was accomplished with 1 mol/L ammonium sulfate, and the flow through was concentrated 10-fold for subsequent gel filtration (Superdex 75 and Superdex Peptide, GE Healthcare). After dialysis, final purification was achieved by means of cation exchange chromatography (Source S, GE Healthcare).

Sera
Sera from patients with peanut allergy, from allergic patients without peanut allergy, and from nonallergic, nonsensitized healthy subjects were included in this study after obtaining informed consent. For detailed characterization, see Table 1 and Table E1 in this article’s Online Repository at www.jacionline.org. The patients refused to have challenge tests with foods to which they knew themselves to be allergic. The sera of patients with allergy but not peanut allergy, and nonallergic, nonsensitized healthy subjects were used as negative controls. The study was approved by the respective local ethics committee (approval no. 10-126).

SDS-PAGE, Western blotting, 2-dimensional PAGE, protein sequencing, and mass spectrometry
For detailed information, see the Methods section in this article’s Online Repository at www.jacionline.org.

Basophil activation test
Basophil activation was determined through expression of CD203c, as previously described, with modifications. Briefly, 100 μL of heparinized whole-blood aliquots of patients with peanut allergy were stimulated with purified allergens in 10-fold serial dilutions starting with 5400 ng/mL and incubated for 20 minutes at 37°C. Nonstimulated cells served as negative controls, and cells stimulated with formyl-methionyl-leucyl-phenylalanine (Sigma-Aldrich, Steinheim, Germany) or zymosan (Sigma-Aldrich) served as positive controls. Afterward, 5 μL of phycoerythrin-conjugated anti-CD203c (Immunotech, Marseille, France) was added to each sample and incubated for an additional 20 minutes at 25°C. Red blood cells were lysed by addition of 2 mL of QuickLysis solution (Medac, Wedel, Germany). After centrifugation (640g for 6 minutes), cells were resuspended in 150 μL of PBS/0.1% azide and 100 μL of 3% paraformaldehyde. Measurements were performed on a flow cytometer (FACSCalibur; BD Biosciences, San Jose, Calif). The mean fluorescence intensity of CD203c on basophils was determined by using FCS Express 4.0 (De Novo Software, Los Angeles, Calif).

Microbial inhibition assays
Antifungal activity assay. Antifungal activity was measured by using a microspectrometric assay, according to the method of Cammue et al. Eighty microliters of fungal spore suspension (2 × 10^5 spores/mL) consisting of Candida albicans, Saccharomyces cerevisiae, Pichia pastoris, Fusarium culmorum, Alternaria species, Cladosporium species, Aspergillus flava, and 20 μL of the synthetic fungal growth medium was supplemented with defined concentrations of peanut defensin on a microplate (final concentration, 0-100 μg/mL). Cultures were incubated for up to 168 hours at a temperature of 22°C. Density measurements were performed at different time points at 570 nm by using a microplate Dynex MRX Revelation Microplate Reader (MTX Lab Systems, Vienna, Va). Percentage growth inhibition was defined as 100 times the ratio of the corrected absorbance of the control microculture minus the corrected absorbance of the test microculture over the corrected absorbance of the control microculture.

Antibacterial activity assay. Microsusceptibility analysis was performed to determine the minimal inhibitory concentration of the tested peanut defensins (up to a concentration of 64 μg/mL) against bacteria of the strains Escherichia coli WBB01 and Staphylococcus aureus (clinical isolate). A 96-well flat-bottom microtiter plate (Corning, Corning, NY) was prepared with the buffer (20 mmol/L HEPES, pH 5.2 and pH 7.4) and serial peptide dilutions to be tested (90 μL). Finally, 10 μL of the bacterial suspension (1 × 10^6 colony-forming units mL−1) in LB broth was added to all but the negative control. Buffer and bacterial suspension served as a positive control, and buffer and growth medium served as a negative control. After incubation at 37°C and 150 rpm, overnight density measurements of the microtiter plates were carried out after 20 hours of inoculation with an ELISA reader (Rainbow; Tecan, Crailsheim, Germany) at 620 nm. The minimal inhibitory concentration was determined as the peptide concentration at which no culture growth was detected.

RESULTS
Detection and isolation of novel peanut allergens
To detect and isolate allergens in the low-molecular-weight range of peanut extract, we used the protocol for the isolation of Ara h 8 from lipophilic extraction under alkaline conditions. When we removed the contaminants by using hydrophobic interaction chromatography, we identified in the flow through some known peanut allergens, such as Ara h 2. Additionally, an intensive protein spot of about 10, 11, and 12 kDa were detectable under nonreducing conditions, whereas under reducing conditions, only 1 band appeared, with a molecular mass of about 8 kDa. The isolated components were analyzed for IgE reactivity by using Western blotting. Sera of patients with peanut allergy, patients with allergy but not peanut allergy, and nonallergic, nonsensitized healthy subjects were screened for IgE reactivity to the proteins separated under nonreducing and reducing conditions.
TABLE I. Clinical data of patients with peanut allergy

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Total IgE (IU/mL)</th>
<th>Peanut extract (kU/L)</th>
<th>Ara h 2 (kU/L)</th>
<th>Ara h 8 (kU/L)</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>M</td>
<td>47</td>
<td>508</td>
<td>&gt;100</td>
<td>60.5</td>
<td>17.3</td>
<td>Anaphylactic shock in childhood, cardiac symptoms after ingestion of low peanut concentrations</td>
</tr>
<tr>
<td>P2</td>
<td>M</td>
<td>14</td>
<td>732</td>
<td>&gt;100</td>
<td>96.5</td>
<td>0.09</td>
<td>Vomiting, urticaria, problems breathing through the nose, tussive irritation, contact urticaria</td>
</tr>
<tr>
<td>P3</td>
<td>F</td>
<td>5</td>
<td>367</td>
<td>71.0</td>
<td>41.0</td>
<td>0.1</td>
<td>No ingestion, skin contact → worsening of atopic eczema</td>
</tr>
<tr>
<td>P4</td>
<td>F</td>
<td>21</td>
<td>1828</td>
<td>47.50</td>
<td>28.9</td>
<td>11.4</td>
<td>Swelling of mucosa of mouth, throat, and larynx; swallowing problems; dyspnea; hypotonia; tremor</td>
</tr>
<tr>
<td>P5</td>
<td>M</td>
<td>42</td>
<td>175</td>
<td>32.9</td>
<td>18.9</td>
<td>0.1</td>
<td>After mere contact: contact urticaria with subsequent OAS, laryngeal edema, maximal fatigue, dyspnea</td>
</tr>
<tr>
<td>P6</td>
<td>M</td>
<td>41</td>
<td>555</td>
<td>19.5</td>
<td>1.15</td>
<td>0</td>
<td>Facial edema, dyspnea, urticaria</td>
</tr>
<tr>
<td>P7</td>
<td>M</td>
<td>12</td>
<td>22.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>OAS to peanut and seasonal rhinoconjunctivitis</td>
</tr>
<tr>
<td>P8</td>
<td>F</td>
<td>35</td>
<td>72.1</td>
<td>0.51</td>
<td>0</td>
<td>7.46</td>
<td>Itching of throat, difficulties in breathing after ingestion of fresh peanuts</td>
</tr>
<tr>
<td>P9</td>
<td>F</td>
<td>38</td>
<td>793.1</td>
<td>5.75</td>
<td>1.17</td>
<td>9.16</td>
<td>OAS to peanut and seasonal rhinoconjunctivitis</td>
</tr>
<tr>
<td>P10</td>
<td>F</td>
<td>23</td>
<td>689.7</td>
<td>1.66</td>
<td>0</td>
<td>13.30</td>
<td>OAS to peanut and seasonal rhinoconjunctivitis</td>
</tr>
<tr>
<td>P11</td>
<td>M</td>
<td>27</td>
<td>536</td>
<td>75.9</td>
<td>150.0</td>
<td>6.13</td>
<td>Anaphylactic symptoms, angioedema, larynx edema, flush, generalized urticaria, dyspnea, absence</td>
</tr>
<tr>
<td>P*</td>
<td>F</td>
<td>51</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>No allergic symptoms</td>
</tr>
</tbody>
</table>

F, Female; M, male; OAS, oral allergy syndrome; P*, control subject.

Functional tests

Whole-blood samples of 12 patients were selected to perform the CD203c basophil activation test, a test acknowledged as a functional assay for allergenicity, to determine whether the IgE-reactive proteins can elicit histamine release. Functional tests demonstrated that the isolated proteins are IgE reactive but lose their IgE-binding capacity on reduction.

Biochemical and physicochemical characterization

Protein bands III (12 kDa), I (11 kDa), and II (10 kDa), as shown in Fig 2, A, were further analyzed by means of protein sequencing, and the 17 N-terminal amino acids were determined. Fig 5 summarizes the results obtained from protein sequencing, homology screening, and mass spectrometric analysis.

Searching for homologous proteins in the National Center for Biotechnology Information protein database was not successful.
However, searching the expressed sequence tags (EST) database and deducing the amino acid sequence from the DNA data, we found complete identity with the sequences of the peanut defensins (except the cysteine residues in amino acid positions 3 and 14 that are not determinable by using Edman degradation).

The 12-kDa band referred to accession number EY396089, the 11-kDa band referred to accession number EY396019, and the 10-kDa protein referred to accession number EE124955. Although the first 2 proteins were very similar among each other, with only 3 amino acid exchanges, the last sequence, EY396089, differed by 27 amino acids (43% sequence identity; Fig 5, A).

Additionally, the masses of the isolated intact proteins were determined by using high-resolution mass spectrometry and compared with the calculated masses (Fig 5).

The spectrum shows 5 main peaks with monoisotopic molecular masses of 5184.1, 5200.0, 5216.1, 5442.4, and 5472.4 Da. The peaks marked as I, II, and III showed in each case an 8-Da lower mass than calculated. The masses corresponding to peaks IV and V were 16 Da and 32 Da higher than peak III. Furthermore, trypic mass fingerprinting was performed with the 3 bands from the gel run under nonreducing conditions to confirm the data. The trypic fragments correspond with the respective sequences that are highlighted in gray in Fig 5, A. The percentages of sequence coverage are 46.8% for EY396019 and EE124955 and 73.9% for EY396089.

A sequence alignment of the peanut defensins and several selected other plant defensins are shown in Fig E3 in this article’s Online Repository at www.jacionline.org.

**DISCUSSION**

Occasionally, the clinical diagnosis of peanut allergy cannot be confirmed by means of extract-based IgE detection and ascribed to known allergens, which indicates the necessity to improve in vitro diagnostic procedures. In the process of purifying Ara h 8 for use in investigations of whether lipid association renders Ara h 8 resistant to temperature and digestion, additional low-molecular-weight and hitherto unidentified IgE-reactive peanut proteins became visible. We pursued the identification and functional characterization of these novel allergens because other sources have shown lipophilic allergens to be clinically relevant and even associated with severe reactions but nonetheless still lacking in routine allergy diagnostic extracts.

The lipophilic extraction strategy was successful in identifying 2 novel allergens and 1 additional isoform in peanuts. They belong to pathogenesis-related protein family 12, the plant defensins. Pathogenesis-related proteins are characterized by the fact that they are synthesized particularly under stress conditions. Our data were submitted to the World Health Organization/International Union of Immunological Societies allergen nomenclature subcommittee, and the peanut allergens were classified as Ara h 12 and 13.

Defensins are small amphiphilic cationic proteins found in plants, invertebrates, and vertebrates. The primary structure of plant defensins is divergent, but plant defensins share common structural characteristics, such as the conserved cysteine spacing pattern and the formation of 4 disulfide bonds between C1-C8, C2-C5, C3-C6, and C4-C7. The obtained 3-dimensional structures are similar to those of a triple-strand anti-parallel β-sheet and one α-helix as characterized on Rs-AFP2, which serves as a model defensin. The same structural elements can be found in insect defensins and scorpion toxins.

We detected the peanut defensins in the low-molecular-weight range after separation from the hydrophobic proteins found in a lipophilic peanut extract at alkaline pH. This fractionation resulted in the protein pattern shown in Fig 1.
Biochemical studies were performed by using protein sequencing and mass spectrometry and resulted in the identification of 2 groups of peanut defensins with sequence identities of only 43% to 45%. The protein masses calculated from the EST sequences were exactly 8 mass units higher than the masses determined for the 3 proteins of the isolated fraction. This suggests the formation of 4 disulfide bonds resulting from the loss of 8 hydrogen atoms. Furthermore, the existence of these defensin molecules in the plant was clearly confirmed by the peptides identified by means of tryptic mass fingerprinting. Although a putative N-glycosylation site is predicted for the sequence of Ara h 12 and Ara h 13, our mass spectrometric results prove that the defensins are not glycosylated, which is in accordance with the literature.

The analysis of Ara h 12 revealed 3 molecular peaks that differed by 16 mass units each. A probable explanation for this is the oxidation of methionine to methionine sulfoxide, which causes an increase of 16 mass units. Ara h 12 contains 2 methionine residues, and thus (besides the nonoxidized form of 5184.1 Da) the masses of 5216.1 Da and 5200.0 Da might be elicited by different numbers of oxidized methionines. In the analysis of Ara h 13, we identified 2 isoforms referring to the EST sequences EY396019 and EE124955. The isoforms differ by 3 amino acids (E2V, S17T, and K46N).
Interestingly, the 3 peanut defensins exhibited unusual migratory behavior in SDS-PAGE. The molecular mass appears to be higher under nonreducing conditions (10, 11, and 12 kDa) than under reducing conditions (8 kDa). Because of disulfide formations, a more compact structure with smaller apparent size could be assumed. However, the peanut defensins probably form dimers under natural conditions similar to what has been described for a tobacco defensin.\textsuperscript{18} Dimerization might also be the reason that the small molecules are able to cross-link surface-bound IgE and activate basophils. In addition, we observed no IgE reactivity in Western blot analysis for sera from patients P11, P12, and P13, but we did observe basophil activation after stimulation with defensins. This might be due to the fact that the sensitivity of the basophil activation test is higher than that of serum IgE detection in the Western blot.\textsuperscript{22,23} Taking into account that the conformation of the defensins is critical for IgE detection, some epitopes might not be detectable in Western blot analysis, resulting in loss of IgE recognition.

IgE reactivity to the peanut defensins was detected in particular in sera from patients with severe anaphylactic reactions to peanut. Studies are already in progress to elucidate whether peanut defensins can be considered marker allergens for severe peanut allergy, as suggested by our preliminary results.

To date, only 3 defensin-related proteins have been described as allergens. Two pollen allergens with a defensin-like domain have already been described in the published literature, Art v 1 from mugwort and Amb a 4 from ragweed.\textsuperscript{24,25} These proteins consist of a 57-amino-acid N-terminal defensin-like domain and a C-terminal, proline-rich, highly glycosylated part of 52 amino acid residues. Thus IgE reactivity cannot be ascribed solely to the defensin part. Additionally, an 8-kDa protein was detected in soybean hull dust that was associated with respiratory symptoms in exposed allergic workers.\textsuperscript{26} The N-terminus of 19 amino acids was determined and showed similarity to defensins. Many other plant defensins have been described, and their sequences are available in databases, but they have not been investigated in relation to allergy. Of all these defensins, Ara h 12 showed the highest sequence similarity to the pea protein Psd1,\textsuperscript{27} with 72% identity, and Ara h 13.0101 showed the highest sequence similarity to the \textit{Vicia faba} protein AC102060, with 70% identity.

Because of the specific defensin structure, the loss of the disulfide formations is probably the reason for the lack of IgE reactivity of the peanut defensins under reducing conditions. However, the disulfide bonds are responsible for the stability of defensins regarding pH changes, proteolytic digestion by trypsin, and extreme temperature changes.\textsuperscript{28} These features might be modulating factors for immunogenicity and could add to the allergenicity of peanut defensins.

Because we discovered that the 2 novel peanut allergens were plant defensins, we were interested in their effect on microbes. Defensins are widely distributed host defense peptides that mainly exhibit antimicrobial activity directed against bacteria, fungi, and viruses. Defensins in seeds of leguminous plants (Fabaceae; eg, peanuts and beans) are part of their innate immune response together with other antimicrobial peptides, such as lipid
transfer proteins, chitinases, chitinase-like proteins, hemolysins, and lectins, some of which are already known to be allergens as well. Most of the Fabaceae-derived defensins show antifungal activity, which is why the investigation of the antimicrobial activity of the peanut defensins started along that line.

A clear growth inhibition of the fungal species *Alternaria* and *Cladosporium* was demonstrated. These results reflect the real situation with the natural mixture of different peanut defensins. Our observations corroborate the results of other research groups, who have also described antifungal activity of plant defensins on *Alternaria* and *Cladosporium* species. Garcia-Olmedo et al. summarize that plant defensins show mostly antifungal activity, whereas lipid transfer proteins are directed against bacteria. Together, these 2 groups of defense proteins form a general barrier against pathogens.

The mechanism through which defensins act is not completely understood. Because of many cationic and hydrophobic amino acids, the defensins can attach to negatively charged membranes. Different groups of defensins can act together.

We were able to identify 2 novel peanut allergens that in our group of patients are IgE reactive, particularly in sera of patients with severe peanut allergy. In addition, we could show antifungal but not antibacterial activity of the peanut defensins. With regard to human subjects, the peanut defensins are a double-edged sword: on the one hand, the antimicrobial effect could be beneficial when patients with atopic dermatitis apply peanut oil–based ointments, but on the other hand, the defensins are allergens possibly associated with severe peanut allergy and are also small enough to penetrate the epidermal barrier, as is known to be the case for other peanut allergens.

The excellent technical support of Marisa Böttger and Barbara Fitz is gratefully acknowledged. Furthermore, we thank the fluorescence core facility at the Research Center Borstel for their assistance.

Clinical implications: Peanut defensins are identified as novel allergens (Ara h 12 and 13) and recognized in particular by patients with severe reactions. They were shown to elicit antifungal but not antibacterial activity.

REFERENCES


METHODS

SDS-PAGE and Western blotting

SDS-PAGE was performed according to the method of Laemmli\(^1\) by using the XCELL Mini Cell System (Novex, San Diego, Calif) with 4-12% or 12% acrylamide Bis-Tris gels. After protein separation, gels were fixed for 30 minutes in 5% glutaraldehyde and afterward stained with 0.2% Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany).

For immunoblot analysis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Schwalbach, Germany) by means of semidyblotting for 45 minutes at 0.8 mA/cm\(^2\), as described previously.\(^2\) Membranes were blocked with 5% skimmed milk powder in Tris-buffered saline with Tween 20 (TTBS; 100 mmol/L Tris, 100 mmol/L NaCl, 2.5 mmol/L MgCl\(_2\), and 0.05% Tween 20 [pH 7.4]) overnight. Patients’ sera were diluted 1:10 with 2.5% skimmed milk powder in TTBS and incubated overnight. Bound antibodies were detected by using a horseradish peroxidase-conjugated secondary antibody, mouse anti-human IgE (Fc)-horseradish peroxidase (1:5000; Southern Biotech, Birmingham, Ala) diluted in TTBS for 2 hours.

Stained blots were detected with the ChemiDoc MP System (Bio-Rad Laboratories). Protein staining on PVDF membranes was accomplished by using India ink (Winsor and Newton, London, United Kingdom) at a dilution of 1:1000 in TTBS for 2 hours or staining with 10% trichloroethanol (Sigma-Aldrich) in 1:1 (vol/vol) water/methanol.\(^3\)

Two-dimensional PAGE

Two-dimensional electrophoresis was performed, as previously described, with slight modifications.\(^4\) Briefly, immobilized pH gradient strips 3-10 (Novex IPG Zoom Strips; Invitrogen, Groningen, The Netherlands) were used for separation in the first dimension (isoelectric focusing). About 100 μg of peanut extract was loaded per gel strip, and the separation was performed at 2 mA and 2 W, with increasing voltage at 200 V for 20 minutes, 450 V for 15 minutes, 750 V for 15 minutes, and 2000 V for 1 hour. SDS-PAGE was carried out at 200 V and 30 mA in the second dimension by using 4-12% acrylamide gradient Bis-Tris gels (Invitrogen). After protein separation, gels were fixed for 30 minutes in 5% glutaraldehyde.

Molecular masses and isoelectric points were determined by comparison with spectra multicolor low-range protein standard (1.7-40 kDa; Thermo Scientific, Vilnius, Lithuania) and Isoelectric Focusing Marker 3-10, Liquid Mix (Serva). Afterward, the gels were Coomassie stained or blotted onto PVDF membrane and immunostained.

Protein sequencing

After blotting, the PVDF membrane was washed with Milli-Q water, stained with 0.1% Coomassie in 50% methanol, destained in 50% methanol, and air-dried. Protein bands were excised, and microsequencing was performed on a Precise protein sequencer with an online phenylthiohydantoin amino acid analyzer (PE Biosystems, Weiterstadt, Germany).\(^5\)

Mass spectrometry

The masses of the protein fraction were analyzed by using a high-resolution electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometer (APEX-Qc; Bruker Daltonics, Billerica, Mass) equipped with a 7 Tesla actively shielded magnet and an Apollo ion source.\(^6\) The tryptic peptides were dissolved in a 50:50:0.001 (vol/vol/vol) mixture of methanol, water, and acetic acid and sprayed with a flow rate of 2 μL/min. For a straightforward interpretation of the heterogeneous samples, the obtained positive ion mass spectra were charge deconvoluted. Mass numbers refer to the monoisotopic mass of the neutral molecules.

Tryptic mass fingerprinting was performed, as described previously.\(^7\) Briefly, Coomassie-stained protein bands were excised, destained, and digested overnight with trypsin (Trypsin Gold, Mass Spectrometry Grade; Promega, Mannheim, Germany), as described previously.\(^8\) Afterward, the corresponding tryptic fragments were mixed 1:1 (vol/vol) with saturated a-cyano-4-hydroxycinnamic acid matrix solution (Bruker Daltonics, Bremen, Germany) and spotted on the target. The samples were analyzed with a Reflex III (Bruker Daltonics) in reflector mode applying an acceleration voltage of 20 kV. External mass calibration was performed with an appropriate mixture of peptides. Mass spectrometric data were analyzed with BioTools 2.0 (Bruker Daltonics).

REFERENCES

FIG E1. Western blot experiments with 14 additional subjects (for characterization, see Table E1) under nonreducing conditions. C, Buffer control; I, India ink staining; M, molecular mass marker; P, patients’ sera (numbers are as listed in Table E1); P4, positive control patient (see Table I); TCE, protein staining with trichloroethanol.
Additional basophil activation tests (CD203c) were performed with 2 patients with peanut allergy, 5 patients with allergy but not peanut allergy, and 1 nonallergic nonsensitized subject. Blood samples were stimulated with 0.0054 to 5,400 μg/L peanut defensin or 4.37 to 437.55 μg/L formyl-methionyl-leucyl-phenylalanine (fMLP) or 5,000 to 500,000 μg/L zymosan as a positive control. For unstimulated samples, PBS was used. MFI, Mean fluorescence intensity; PE, phycoerythrin.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Species</th>
<th>Allergen</th>
<th>Protein Accession Number</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Raphanus sativus</em></td>
<td>radish</td>
<td></td>
<td>AAA69540</td>
<td>QKLQCRSPCTWG-QVCSBN-NAC-CKQK1REKAKHSGCNVYFP-AHKC1GYFPC</td>
</tr>
<tr>
<td><em>Arachis hypogaea</em></td>
<td>peanut</td>
<td>Ara h 12</td>
<td>EY396089*</td>
<td>KLCNLADTYK-GPCFT---NACD---HCKNKHEKHYSTGTCNK---NA-CHCAHNC</td>
</tr>
<tr>
<td><em>Arachis hypogaea</em></td>
<td>peanut</td>
<td>Ara h 13.0101</td>
<td>EY396019*</td>
<td>KFCNLSFKPK-GPCFT---KSKCD---HKCROI-HHLLGGCRLDDFR-CHCNRRKC</td>
</tr>
<tr>
<td><em>Arachis hypogaea</em></td>
<td>peanut</td>
<td>Ara h 13.0102</td>
<td>EE124955*</td>
<td>KVCNLSFKPK-GPCFT---TKCDD---HICKDD-HHLLGGCRLDDFR-CCHCNRRKC</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>soybean</td>
<td>Gly m 2</td>
<td>A57106</td>
<td>YTITENLPAITRLLGPMXY</td>
</tr>
<tr>
<td><em>Artemisia vulgaris</em></td>
<td>mugwort</td>
<td>Art v 1</td>
<td>Q84Z5X5</td>
<td>AGS KelcKETBKTG-GKCDN----KKCDK-KC1SDDKAQHGACIHREAGKESCYFDDCSC</td>
</tr>
<tr>
<td><em>Ambrosia artemisiifolia</em></td>
<td>ragweed</td>
<td>Amb a 4</td>
<td>CBK52317</td>
<td>KLCEKPSVTWK-GKCVGTPCDK-HC1XKEAKHGCAXKRRD-KATCFYFEDDC</td>
</tr>
<tr>
<td><em>Pisum sativum</em></td>
<td>pea</td>
<td></td>
<td>P81929</td>
<td>KTCENLADTFK-GPCFT---NACD---HCKNKHEKHYSTGTCNK---NK-CKCTQNC</td>
</tr>
<tr>
<td><em>Vicia faba</em></td>
<td>broad bean</td>
<td></td>
<td>AC102060</td>
<td>KTCENLADTFK-GPCFT---GDDCNK-HCKNKHEKHYSTGTCNK---CHGRCR</td>
</tr>
<tr>
<td><em>Arachis hypogaea</em></td>
<td>peanut</td>
<td></td>
<td>Q2PXN3</td>
<td>ATCENLADTFK-GPCFT---GDDCNK-HCKNKHEKHYSTGTCNK---CHGRCR</td>
</tr>
<tr>
<td><em>Arachis diogoi</em></td>
<td>peanut</td>
<td></td>
<td>Q67FY4</td>
<td>ATCENLADTFK-GPCFT---MKCDN---HKCKDENLGGCRLDDFR---CHGRCRC</td>
</tr>
<tr>
<td><em>Arachis diogoi</em></td>
<td>peanut</td>
<td></td>
<td>AAO72633</td>
<td>KTCENLADTFK-GPCFT---GDDCNK-HCKNKHEKHYSTGTCNK---CHGRCR</td>
</tr>
</tbody>
</table>

* accession number from EST database

**FIG E3.** Sequence alignment of selected plant defensins. *Rs-AFP2* is the radish (*Raphanus sativus*) plant defensin.\textsuperscript{15} Defensins from the Leguminosae include Ara h 12-13 (peanut defensins), pea (*Pisum sativum*), Gly m 2 from *Glycine maxima* (only the N-terminal sequence is known\textsuperscript{15}). Plant allergens with defensin-like domains include Art v 1 from *Artemisia vulgaris*\textsuperscript{15} and Amb a 4 from *Ambrosia artemisiifolia*\textsuperscript{15,16}. Amino acids conserved in plant defensins are indicated in red; mostly conserved amino acids are in blue.
TABLE E1. Clinical data of additional patients with peanut allergy and control subjects

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Total IgE (IU/mL)</th>
<th>Peanut extract (kU/L)</th>
<th>Ara h 2 (kU/L)</th>
<th>Ara h 8 (kU/L)</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>P12</td>
<td>M</td>
<td>19</td>
<td>260.00</td>
<td>66.30</td>
<td>22.00</td>
<td>1.79</td>
<td>Dyspnea, flush, conjunctivitis, facial edema</td>
</tr>
<tr>
<td>P13</td>
<td>F</td>
<td>24</td>
<td>92.70</td>
<td>18.0</td>
<td>14.0</td>
<td>4.08</td>
<td>Generalized urticaria, massive generalized itching, conjunctivitis</td>
</tr>
<tr>
<td>P14</td>
<td>F</td>
<td>37</td>
<td>281</td>
<td>1.03</td>
<td>0</td>
<td>2.91</td>
<td>Allergic, pollen-associated food allergy but no symptoms after peanut consumption</td>
</tr>
<tr>
<td>P15</td>
<td>F</td>
<td>51</td>
<td>311.3</td>
<td>3.54</td>
<td>0.00</td>
<td>10.50</td>
<td>Pollen-associated food allergy but no symptoms after peanut consumption</td>
</tr>
<tr>
<td>P16</td>
<td>F</td>
<td>21</td>
<td>128.1</td>
<td>2.31</td>
<td>0</td>
<td>10</td>
<td>Allergic, specific peanut IgE against peanut extract and Ara h 8, no symptoms after consumption</td>
</tr>
<tr>
<td>P17</td>
<td>F</td>
<td>49</td>
<td>142</td>
<td>0.4</td>
<td>0</td>
<td>3.08</td>
<td>Allergic, specific peanut IgE against peanut extract and Ara h 8, no symptoms after consumption</td>
</tr>
<tr>
<td>P18</td>
<td>M</td>
<td>26</td>
<td>49.3</td>
<td>0</td>
<td>0</td>
<td>5.07</td>
<td>Pollen-associated food allergy but no symptoms after peanut consumption</td>
</tr>
<tr>
<td>P19</td>
<td>F</td>
<td>39</td>
<td>87.3</td>
<td>0</td>
<td>0</td>
<td>3.44</td>
<td>Pollen-associated food allergy but no symptoms after peanut consumption</td>
</tr>
<tr>
<td>P20</td>
<td>F</td>
<td>30</td>
<td>138.5</td>
<td>0</td>
<td>0</td>
<td>8.48</td>
<td>Pollen-associated food allergy, specific peanut IgE against Ara h 8, no definite allergic symptoms after consumption (once minor perioral dermatitis [minuscule papules], excluding the lips): suspected irritant contact dermatitis</td>
</tr>
<tr>
<td>P21</td>
<td>F</td>
<td>47</td>
<td>46.10</td>
<td>0</td>
<td>0</td>
<td>1.4</td>
<td>Pollen-associated food allergy but not peanut allergy (no symptoms after peanut consumption)</td>
</tr>
<tr>
<td>P22</td>
<td>F</td>
<td>59</td>
<td>161.9</td>
<td>0</td>
<td>0</td>
<td>4.84</td>
<td>Pollen-associated food allergy but no symptoms after peanut consumption</td>
</tr>
<tr>
<td>P23</td>
<td>F</td>
<td>53</td>
<td>3.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Allergic (eg, birch pollen allergy) but no symptoms after peanut consumption</td>
</tr>
<tr>
<td>P24</td>
<td>M</td>
<td>70</td>
<td>11.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>No allergic symptoms</td>
</tr>
<tr>
<td>P25</td>
<td>M</td>
<td>47</td>
<td>5.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>No allergic symptoms</td>
</tr>
</tbody>
</table>

F, Female; M, male.