

Peanut defensins: Novel allergens isolated from lipophilic peanut extract

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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;■■■:■■■-■■■.)

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.¹ 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,² especially in US and European children.³ 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 proteins⁴ (ie, dry roasting of peanuts or roasting them in oil for variable times and temperatures^{5,6} 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. Becker⁷ 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%,⁸ lipophilic allergens might not be included in aqueous extracts.⁹ For example, Leduc et al¹⁰ 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.⁸ 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.¹¹ 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

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Abbreviations used

EST: Expressed sequence tags
PVDF: Polyvinylidene fluoride
TTBS: Tris-buffered saline with Tween 20

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.¹¹ 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.¹² Briefly, 100 μ L 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 μ L 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 μ L of PBS/0.1% azide and 100 μ L 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.¹³ Eighty microliters of fungal spore suspension (2×10^4 spores/mL) consisting of *Candida albicans*, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Fusarium culmorum*, *Alternaria* species, *Cladosporium* species, *Aspergillus flavus*, and 20 μ L of the synthetic fungal growth medium was supplemented with defined concentrations of peanut defensin on a microplate (final concentration, 0–100 μ g/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 μ g/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 μ L). Finally, 10 μ L of the bacterial suspension (1×10^6 colony-forming units $\cdot$ 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 8 kDa and an isoelectric point of 7.7 to 8.1 was detectable by using 2-dimensional PAGE under reducing conditions (Fig 1).

Immunologic characterization

SDS-PAGE was performed under nonreducing and reducing conditions to identify and characterize the isolated proteins (Fig 2, A). Fig 2, A, shows that 3 bands 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

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

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

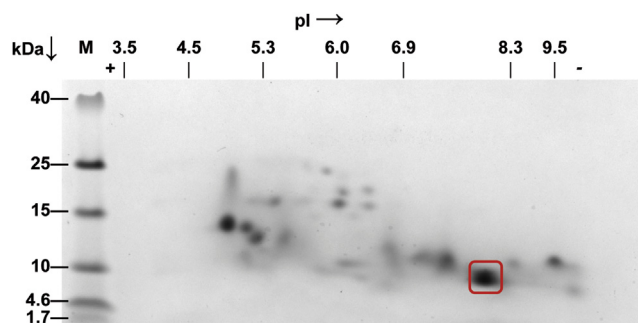


FIG 1. Two-dimensional PAGE of hydrophobic interaction chromatography flow through of the lipophilic peanut extract. Separation was performed under reducing conditions and Coomassie stained. The protein spot under consideration is indicated. M, Molecular mass marker; pI, isoelectric point.

As demonstrated in Fig 2, B, patients P2, P3, and P4 (according to Table I) show strong clear bands, whereas patient P9 shows only weak bands in the range of 10 to 12 kDa. Patients P1 and P5 react only weakly to one of the 3 bands. Additional control sera (P14-P25) showed no IgE reactivity under nonreducing conditions (see Fig E1 in this article's Online Repository at www.jacionline.org). Interestingly, most of the reactive sera were obtained from patients with severe anaphylactic reactions to peanut. The positive sera recognized the 3 proteins with different intensities, although the upper of the 3 protein bands is the most prominent. However, staining with India ink did not detect any protein band in the nonreduced and reduced sample (see Fig 2, B and C, and Fig E1, lane I). Interestingly, only one of the 6 positive patient sera (P2) bound under reducing conditions (Fig 2, C). The results demonstrate that the isolated proteins are IgE reactive but lose their IgE-binding capacity on reduction.

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.¹⁴

Fig 3 depicts the results of the sera (patients P1, P3, P5, and P11) studied with a serial dilution of the allergen (0.0054-5400 µg/mL). Although basophil activation for patients P1, P3, P5, and P11, as well as for patients P12 and P13 (see Fig E2 in this article's Online Repository at www.jacionline.org), was detectable, no basophil activation was observed for patients P17, P20, P21, P22, P23, and P* (see Fig E2).

In parallel, we cultivated several bacterial and fungal species with the peanut defensins and determined the relative inhibition on microbial growth after different time intervals. The bacterial strains *E coli* WBB01 as an example of gram-negative and *Staphylococcus aureus* as an example of gram-positive bacteria showed no effect on the addition of up to 64 µg/mL peanut defensins, which is similar to the yeasts *C albicans*, *P pastoris*, and *S cerevisiae* (data not shown).

In contrast, as shown in Fig 4, a dose-dependent effect was observed for the mold species *Alternaria* and *Cladosporium*: low concentrations (6.25 µg/mL) caused a slight effect or even, in the case of *Alternaria* species, an increase in growth during the first 24 hours of culture, whereas 25 to 100 µg/mL of the peanut defensin showed a prolonged inhibitory effect on fungal growth of about 70% with *Alternaria* and *Cladosporium* species. For *F culmorum* and *A flavus*, slightly decreased cell growth was detectable only during the first 24 to 48 hours of culture (data not shown).

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.

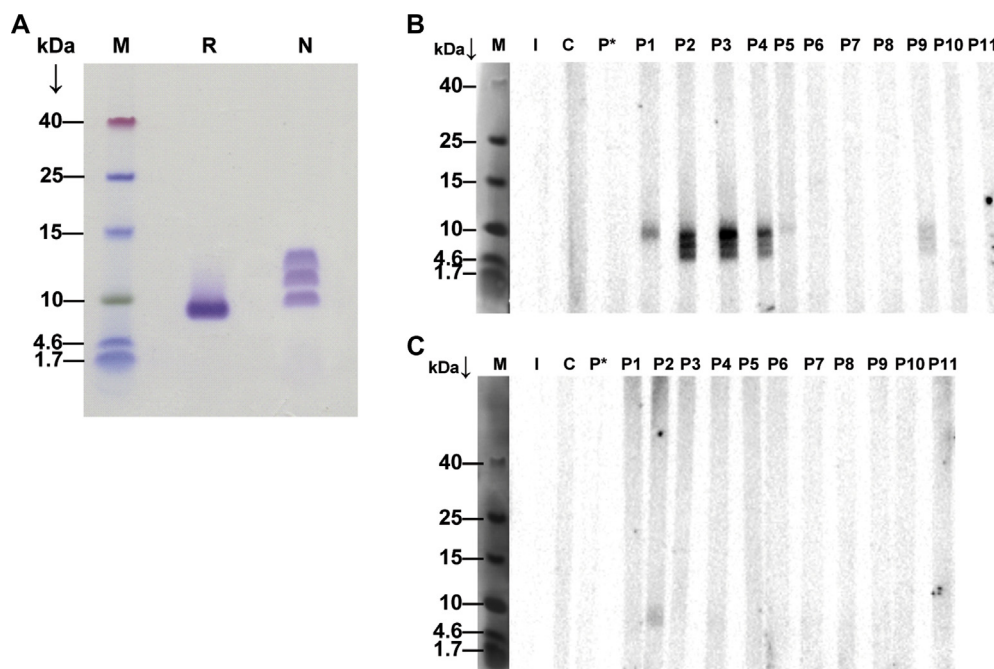


FIG 2. A, SDS-PAGE of the isolated protein fraction separated under reducing (*R*) and nonreducing (*N*) conditions with Coomassie staining. B and C, Western blots for identification of IgE-reactive sera from patients with peanut allergy under nonreducing (Fig 2, B) and reducing (Fig 2, C) conditions. C, Buffer control; I, India ink staining; M, molecular mass marker; P, patients' sera (numbers as listed in Table I); P*, nonallergic subject.

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, tryptic mass fingerprinting was performed with the 3 bands from the gel run under nonreducing conditions to confirm the data. The tryptic 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,¹¹ 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.¹⁵ 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.¹⁶ The primary structure of plant defensins is divergent, but plant defensins share common structural characteristics,¹⁷ such as the conserved cysteine spacing pattern and the formation of 4 disulfide bonds between C1-C8, C2-C5, C3-C6, and C4-C7.¹⁸ 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.¹⁹ The same structural elements can be found in insect defensins and scorpion toxins.^{17,20}

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.

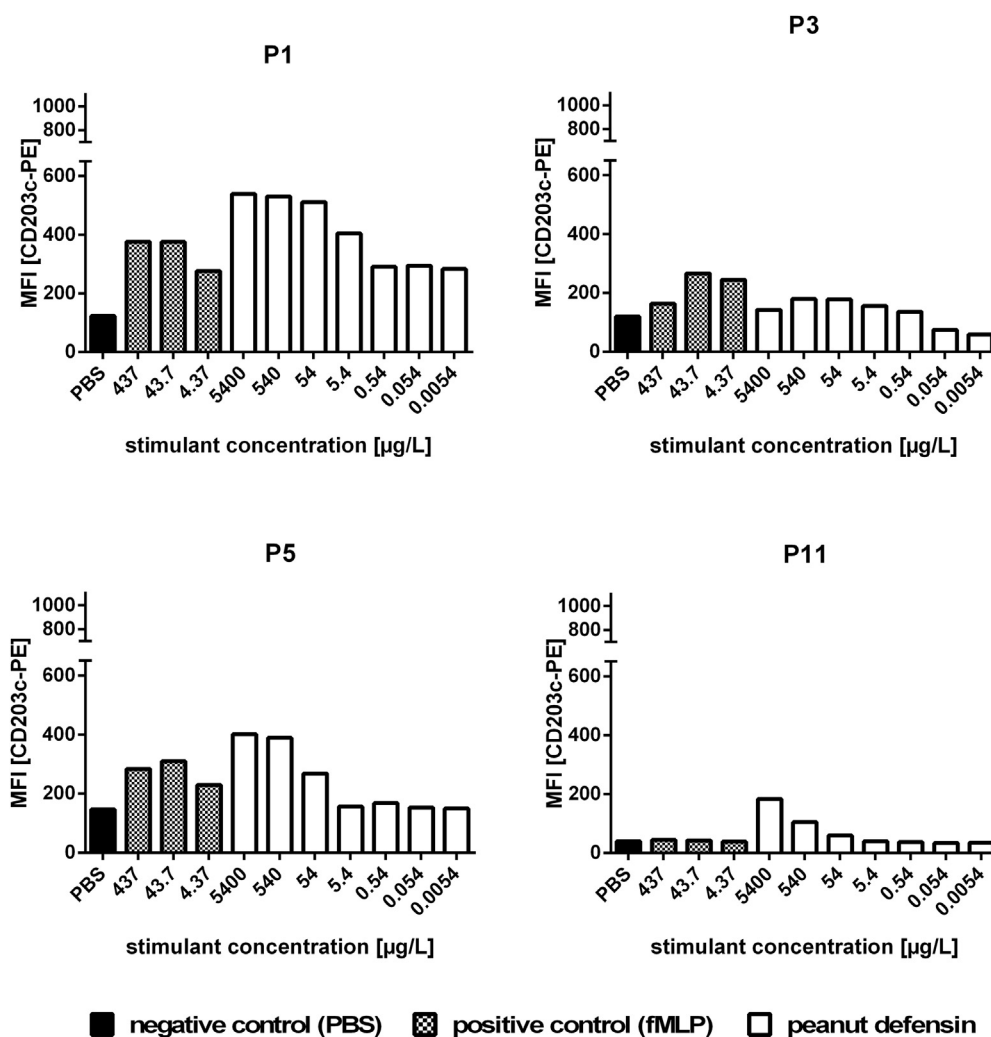


FIG 3. The basophil activation test (CD203c) was performed in blood samples of 4 patients with peanut allergy. Blood samples were stimulated with 0.0054 to 5400 $\mu\text{g/L}$ peanut defensin or 4.37 to 437.55 $\mu\text{g/L}$ formyl-methionyl-leucyl-phenylalanine (fMLP) as a positive control. For unstimulated samples, PBS was used. MFI, Mean fluorescence intensity; PE, phycoerythrin.

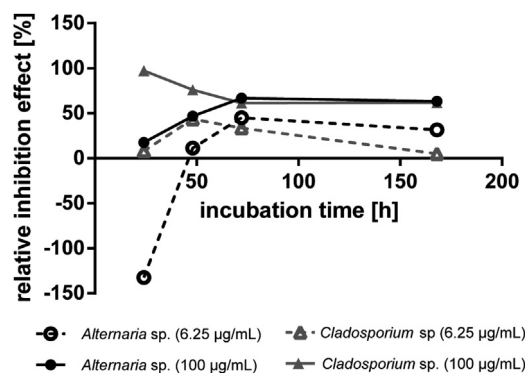


FIG 4. Determination of antifungal activity by using a growth inhibition assay of different fungal species analyzed in the presence of the peanut defensin.

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,²¹ 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).

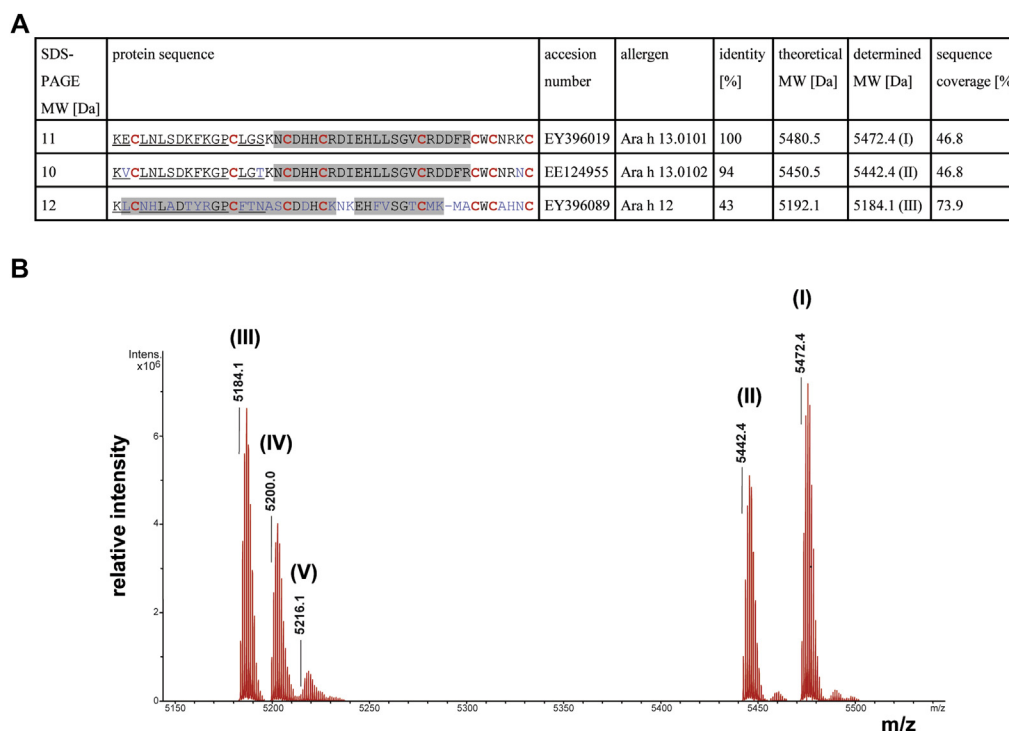


FIG 5. A, Comparison of the 3 peanut defensins detected by using SDS-PAGE under nonreducing conditions. Complete protein sequences were deduced from the EST database. *N*-terminal protein sequences are underlined. Sequence comparison is presented as identity in percentages. Tryptic peptide fragments were analyzed by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and are highlighted in gray. *MW*, Molecular weight. **B,** Electrospray ionization–Fourier transform ion cyclotron resonance mass spectrum of the isolated protein fraction. Monoisotopic neutral masses are indicated.

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.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.^{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.^{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.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 Psd1,²⁷ with 72% identity, and Ara h 13.0101 showed the highest sequence similarity to the *Vicia faba* protein ACI02060, 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.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,²⁸ 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,^{29,30} who have also described antifungal activity of plant defensins on *Alternaria* and *Cladosporium* species. Garcia-Olmedo et al³¹ and Stotz et al³² 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.

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METHODS

SDS-PAGE and Western blotting

SDS-PAGE was performed according to the method of Laemmli^{E1} 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 semidry blotting for 45 minutes at 0.8 mA/cm², as described previously.^{E2} 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 MgCl₂, 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. Immunostaining was performed applying chemiluminescent Western blot detection with Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, Calif). 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^{E3} or staining with 10% trichloroethanol (Sigma-Aldrich) in 1:1 (vol/vol) water/methanol.^{E4}

Two-dimensional PAGE

Two-dimensional electrophoresis was performed, as previously described, with slight modifications.^{E5} 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 Procise protein sequencer with an online phenylthiohydantoin amino acid analyzer (PE Biosystems, Weiterstadt, Germany).^{E6}

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-Qe; Bruker Daltonics, Billerica, Mass) equipped with a 7 Tesla actively shielded magnet and an Apollo ion source.^{E7} The lyophilized proteins 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.^{E7}

Briefly, Coomassie-stained protein bands were excised, destained, and digested overnight with trypsin (Trypsin Gold, Mass Spectrometry Grade; Promega, Mannheim, Germany), as described previously.^{E8} Afterward, the corresponding tryptic fragments were mixed 1:1 (vol/vol) with saturated α-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).

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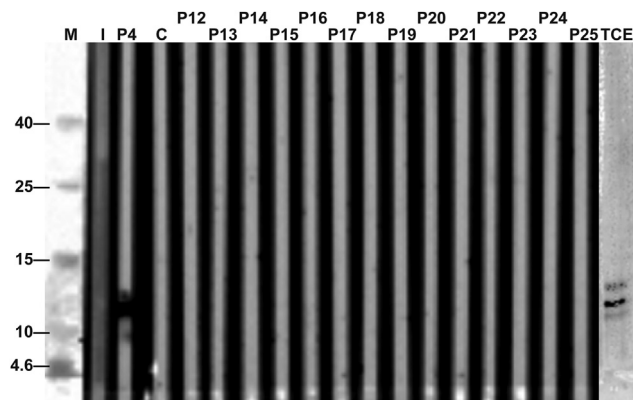
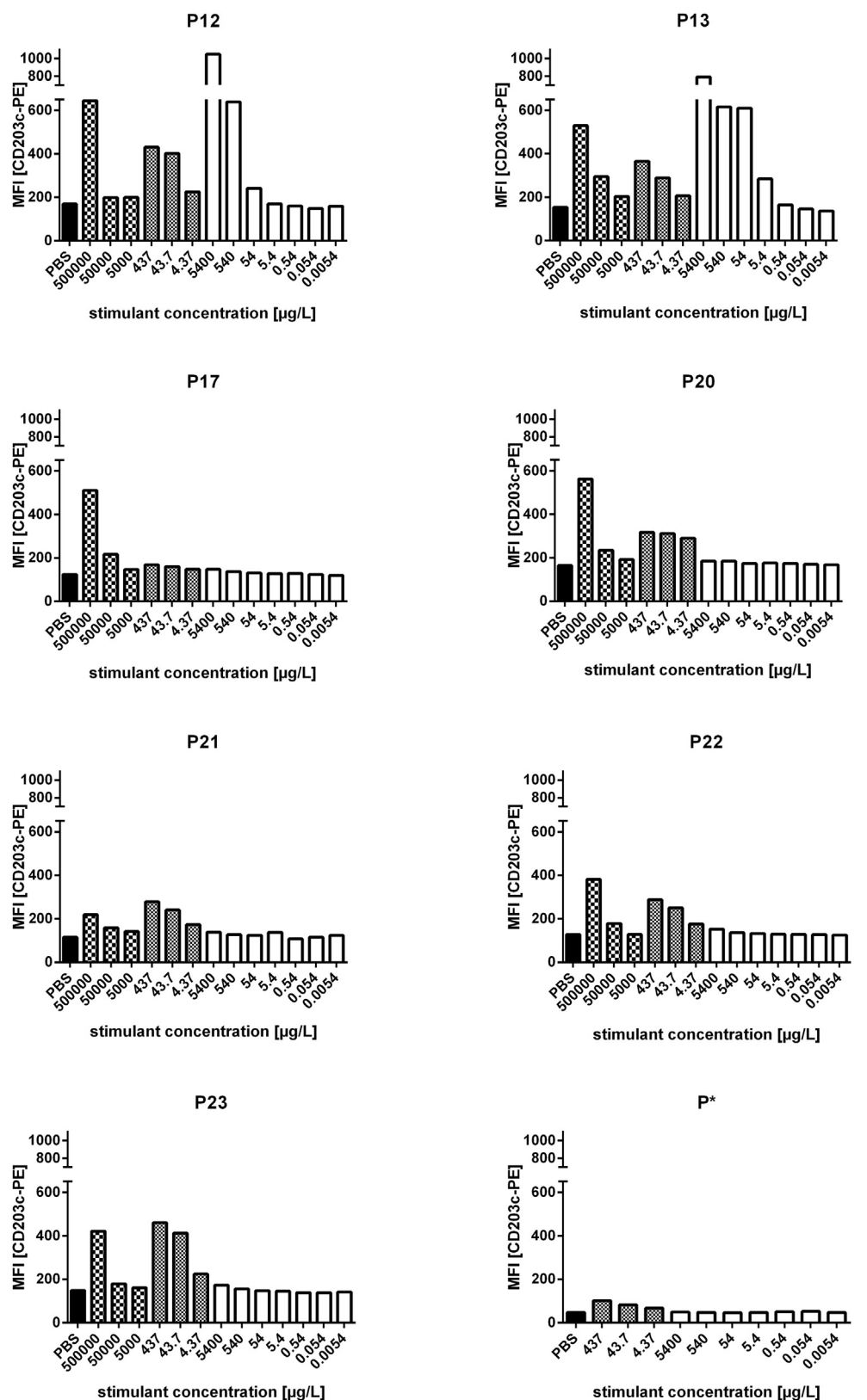


FIG E1. Western blot experiments with 14 additional subjects (for characterization, see [Table E1](#)) under nonreducing conditions.^{E4} C, Buffer control; I, India ink staining; M, molecular mass marker; P, patients' sera (numbers are as listed in [Table E1](#)); P4, positive control patient (see [Table I](#)); TCE, protein staining with trichloroethanol.



■ negative control (PBS) ■ positive control (Zymosan) ■ positive control (fMLP) □ peanut defensin

FIG E2. 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.

Organism	Species	Allergen	Protein Accession Number	Sequence
				1 10 20 30 40 50
<i>Raphanus sativus</i>	radish	-	AAA69540	QKLCQRPSTWS-GVCGNN-NA-C-KNQCIIRLEKARHGSCHNYVFP-AHKCICYFP
<i>Arachis hypogaea</i>	peanut	Ara h 12	EY396089 ^e	KLCNHLADTYR-GPCFT--NASCD-DHCKNKEHFVSGTCMK-MA----CWCANH
<i>Arachis hypogaea</i>	peanut	Ara h 13.0101	EY396019 ^e	KFCNLNLSDFK-GPCLG--SKNCD-HHCRDIEHLLSGVCRDDFR----CWCNRKC
<i>Arachis hypogaea</i>	peanut	Ara h 13.0102	EE124955 ^e	KVCLNLSDFK-GPCLG--TKNCD-HHCRDIEHLLSGVCRDDFR----CWCNRNC
<i>Glycine max</i>	soybean	Gly m 2	A57106	YTITENLPADTYRLGPFXY
<i>Artemisia vulgaris</i>	mugwort	Art v 1	Q84ZX5	AGSKLCEKTSKTYSGKCDN---KKCDK-KCIEWEKAQHGACHKREAGKESCFYFDCSK...
<i>Ambrosia artemisiifolia</i>	ragweed	Amb a 4	CBK52317	KLCEKPSVTWS-GCKVKQTDKCDK-RCIEWEGAKHGACHKRDS-KATCFYFDCDP...
<i>Pisum sativum</i>	pea	-	P81929	KTCEHLADTYR-GVCF--NASCD-DHCKNKAHLISGTCNH-WK----CFCTQN
<i>Vicia faba</i>	broad bean	-	ACI02060	KTCENLSDTFK-GPCI-PD-GNCKN-HCKNNEHLLSGRCRDDFR----CWCTRNC
<i>Arachis hypogaea</i>	peanut	-	Q2PXN3	ATCENLADTYR-GPCFT--TGSCD-DHCKNKEHLLSGRCRDDFR----CWCTRNC
<i>Arachis diogeni</i>	peanut	-	Q67FY4	ATCENLADTFR-GPCFG--NSNCF-HCKTKEHLLSGRCRDDFR----CWCCKRC
<i>Arachis diogeni</i>	peanut	-	AAO72633	KTCENLADKYR-GPCFSGCDTHC-----TTKEHAVSGRCRDDFR----CWCCKRC

^e accession number from EST database

FIG E3. Sequence alignment of selected plant defensins. *Rs-AFP2* is the radish (*Raphanus sativus*) plant defensin.^{E9} Defensins from the Leguminosae include Ara h 12-13 (peanut defensins), pea (*Pisum sativum*), Gly m 2 from *Glycine max* (only the N-terminal sequence is known^{E10}). Plant allergens with defensin-like domains include Art v 1 from *Artemisia vulgaris*^{E11} and Amb a 4 from *Ambrosia artemisiifolia*.^{E12} 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

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

F, Female; M, male.