Published in final edited form as:

Clin Exp Allergy. 2009 July ; 39(7): 1099–1108. doi:10.1111/j.1365-2222.2009.03273.x.

Effector Activity of Peanut Allergens: A critical role for Ara h 2, Ara h 6, and their variants

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Abstract

Rationale—An important property of allergens is their ability to cross-link IgE and activate mast cells and basophils. The effector activity of peanut allergens has not been well characterized.

Methods—Crude extracts of fresh peanut flour were fractionated by gel filtration. Effector function was assayed by measuring degranulation of RBL SX-38 cells sensitized with IgE from individual sera and from pools of sera of peanut allergic donors.

Results—Following gel filtration, 75±7% of the applied protein and 76±16% (n=3) of the applied activity (assayed with a pool of 11 sera) were recovered in the resultant fractions. The majority (85±2%; n=3) of the recovered activity resided in a fraction with a theoretical average molecular weight of ~20 kD and a range of 13–25 kD. When all the individual fractions were recombined, the measured activity was similar to that of the original extract (140±43% when measured with a pool of serum (n=2) and 66±7% when measured with individual sera (n=4)); when all individual fractions excluding the 20 kD fraction were recombined, the measured activity was only 8±2% (n=2) of the original extract when assayed with the serum pool and 10±4% (n=3) when assayed with the individual sera. Two dimensional gel electrophoresis of this biologically active fraction by revealed >60 protein spots. Analysis of 50 of the most prominent spots by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry and of the full mixture by automated tandem mass spectrometry coupled to online capillary liquid chromatography revealed that greater than 97% of the protein mass consisted of Ara h 2.0101, Ara h 2.0201, Ara h 6 isoforms, and variants of these proteins.

Conclusions—Ara h 2 and Ara h 6 account for the majority of the effector activity found in a crude peanut extract.

Keywords

Peanuts; mast cells; basophils; IgE; allergens

Introduction

Hypersensitivity to foods is now recognized as a worldwide problem [1]. Immediate hypersensitivity reactions to peanuts are unusual in that they are much more prevalent and

severe than for other legumes and tend to persist into adulthood [2]. Substantial effort has been made to identify the major peanut allergens in hopes of desensitization of patients specifically to these proteins, production of peanuts without allergenic activity, and the development of skin test reagents using recombinant proteins [3–14]. Nine potentially important allergens of peanut have so far been identified (Ara h 1 - Ara h 8 plus peanut oleosin) [15–20].

The term “major allergen” was originally used by Lowenstein to designate allergens that cause sensitization in >50% of patients and was later redefined by Marsh et al. to include only allergens that elicit an IgE response in >90% of allergic patients [21–23]. The characteristics now widely used to define a “major allergen” are based on those suggested by the WHO/IUIS Allergen Nomenclature subcommittee in 1994 and include: **1) a prominent band** seen with sera from most patients on IgE immunoblots of 1D gels, **2) activity in basophil histamine release (BHR) assays**, **3) activity in competitive ELISA assays**, and **4) activity in animal models** [24]. Finally, **5) the contribution of the allergen to the total potency of the extract should be demonstrated by depletion studies** in which the putatively important allergen is specifically removed from the whole extract and the residual is demonstrated to have reduced activity [23,24]. This fifth criterion aims to address the quantitative contribution a single allergen to the total effector activity of a crude extract. For example, de Groot et al. demonstrated that treating a crude extract of cat dander with anti-Fel d 1 significantly diminished the effector activity of the extract as measured in a basophil histamine release (BHR) assay [25]. Further, purified Fel d 1 contained enough effector activity to explain a substantial amount of the activity in the extract [25]. None of the known peanut allergens have so far satisfied this fifth criterion.

Ara h 1, Ara h 2, and Ara h 3 were identified as major peanut allergens based on the first four criteria but not the fifth [26–29]. Ara h 1 belongs to the vicilin (7S) family of seed storage proteins. Monomers of 63 kD (seen on immunoblots) form stable 150 kD trimers and higher order multimers [30,31]. Ara h 2 belongs to the conglutin family of seed storage proteins and is related to the 2S albumin family [27]. The C-terminus of Ara h 2 has some homology with non-specific lipid transfer proteins (nsLTPs) and with several other peanut proteins, specifically Ara h 6, and Ara h 7. Ara h 3, a peanut glycinin belongs to the legumin (11S) family of seed storage proteins. Native glycinin is a 360–380 kD protein constructed from 60 kD monomeric units [32,33]. Conformational models of Ara h 2 and Ara h 6 are virtually superposable, indicating near identical tertiary structures of the two proteins [34]. Recently, additional isoforms of Ara h 6 have been described and their clinical reactivity demonstrated in large patient populations. These findings indicate that Ara h 6 is also a major allergen [35–37].

In trying to understand the relative contributions of peanut allergens to the overall effector activity of a crude peanut extract, separate groups found that whereas Ara h 1 and Ara h 3 bind IgE strongly, Ara h 2 is much more potent than Ara h 1 or Ara h 3 in degranulation assays [38–40]. However, selective removal of Ara h 2 from an extract of peanuts minimally affects its biologic activity [41]. Finally, Ara h 6, a relatively minor allergen based on its ability to elicit an IgE response, has potent effector activity similar to Ara h 2 [34]. These findings underscore the concept that the ability of an allergen to elicit an IgE response and to bind IgE is not the same as the ability of an allergen to cross-link allergen-specific IgE on mast cells and basophils leading to cell activation.

Here we address the question of which peanut allergens account for the majority of the effector function in highly peanut-allergic individuals and report that most of the effector activity of a saline extract of raw peanuts is due to Ara h 2, Ara h 6, and their variants.

Materials and Methods

Subjects and sera

This study was approved by the Institutional Review Board of the University of Colorado Health Science Center. All subjects signed informed consent. Individuals were selected on the basis of having high concentrations of specific IgE to peanuts and at least 10% of their IgE directed against peanuts. All subjects had a peanut specific IgE > 13 IU/ml on the Pharmacia ImmunoCap assay, a level associated with a 95% confidence level of having clinical reactivity to peanuts [42]. In addition to using individual sera, pools of sera were used. For these, individual patient sera were combined proportionally based on the concentration of anti-peanut IgE. This gave pools with 320 and 375 U/ml of total IgE and 88 and 95 U/ml of anti-peanut IgE; values similar to what we have previously described [41]. The characteristics of the patients and their sera as well as the components of the serum pools are shown in Table 1.

Reagents

Crude Peanut Extracts

Crude peanut extracts were prepared by modification of published protocols [40,43]. Raw Georgia Green peanuts (a gift from Golden Peanut Company, Ashburn, GA, USA) were manually ground with a mortar and pestle while frozen with liquid nitrogen. The ground peanuts were then defatted with diethyl ether in a Soxhlet extractor for 2 hours and dried. The defatted peanut flour was resuspended at 1:5 w/v in ice-cold TBS (150 mM NaCl, 50 mM Tris, pH 7.4) with EDTA-free protease inhibitors (Roche, Nutley, NJ, USA) and mixed overnight at 4°C. Batches of crude peanut extract (CPE) (31–33 mg/ml) were clarified by centrifugation and frozen in aliquots at –70°C. Protein concentrations were determined with the Pierce BCA kit (Pierce, Rockford, IL, USA) using BSA as the standard.

Chromatography

Aliquots of CPE were thawed, spun at 10,000 × g for 10 minutes at 4°C and filtered with a 0.22 µm filter prior to chromatography. Protein concentration was minimally decreased (4%) by this step. Gel filtration was carried out on either a HiLoad 26/60 Superdex 75 Prep-Grade Column (column volume ~325 ml) at 2 ml/min or a Superdex 200 HR 10/30 analytical column (column volume ~24 ml) at 1 ml/min. All runs were performed with an ÄKTA FPLC (GE Healthcare Bio-Sciences AB Uppsala, Sweden). Protein concentrations of the individual peaks were measured with a BCA kit using BSA as a standard and the relative molecular masses of each peak were determined by reference to a calibration curve based on known proteins (*i.e.*, ferritin, 440 kD; BSA, 67 kD; β-lactoglobulin, 35 kD; cytochrome C, 13.6 kD; aprotinin, 6.5 kD; and vitamin B12, 1.3 kD). Samples were aliquotted and frozen at –70° C. Proportional amounts of each fraction were recombined to reconstitute the CPE, both with and without the 20 kD fraction.

Electrophoresis and IgE immunoblots

Both one and two dimensional gel electrophoresis and IgE immunoblots were performed as previously described except that PVDF membranes were used throughout and the 2D immunoblot was developed with biotinylated mouse anti-human IgE and Streptavidin-HRP (both, Invitrogen, Carlsbad, CA)) [41]. Silver staining was performed with a SilverSnap Stain kit (Pierce, Rockland, IL).

RBL SX-38 cell assay and determination of relative EC50 and relative units

RBL SX-38 cells were grown, sensitized, triggered, and the data analyzed as previously described [41]. The specificity, sensitivity, and limitations of this assay have been previously described [39,44]. Spontaneous degranulation (buffer) was $14 \pm 2\%$ ($n=14$; mean $\pm$ SEM) and was subtracted from total degranulation to give the “net degranulation” as previously described [41]. In these experiments ($n=14$), the total degranulation was $56 \pm 2\%$ in the presence of 250 ng/ml of CPE and $61 \pm 2\%$ with 3 μ g/ml of goat anti-human IgE. After subtracting the background for each experiment this gives a net release of $42 \pm 2\%$ with CPE and of $47 \pm 2\%$ with anti-IgE. In these experiments, CPE (250 ng/ml) gave $91 \pm 3\%$ of the net release seen with anti-IgE (3 μ g/ml). For analysis, the maximal net release with an optimal amount of CPE was arbitrarily set to 100% for each experiment so that results from different experiments could be pooled. The EC50 was defined as the concentration of each fraction giving 50% of the degranulation seen with an optimal concentration of CPE (250 ng/ml). Relative units are used to compare the activity of fractions with each other and with the original CPE. Relative units were determined as the reciprocal of the dilution giving the EC50. For example, if a specific chromatographic fraction when diluted 1000 fold gave exactly 50% of the maximal activity seen with CPE, it would contain 1000 relative units. Relative activity of the CPE will vary with each serum or pool of serum due to variations in the amount of IgE, the fraction of the IgE that recognizes peanut proteins, the affinity of that IgE for peanut proteins, and the intrinsic variability of the assay.

Reduction, Alkylation, and Standard Overnight Digestion

Disulfide bonds were reduced by the addition of DTT at a final concentration of 5 mM (pH 7.4 using 25 mM ABC) and incubation for 30 minutes at 70 °C. After cooling to room temperature, IAM was added (15 mM) and the samples were incubated again (in the dark at room temperature, 45 minutes). Proteolysis was carried out by adding trypsin (1:50) to the protein fraction and incubating overnight at 37 °C.

Electrospray Ionization Mass Spectrometry

Samples were analyzed on a LTQ-FT Ultra hybrid mass spectrometer. Peptide desalting and separation were achieved on a dual capillary/nano pump HPLC system (Agilent 1200, Palo Alto, CA). Here, 8 μ L of sample was loaded onto a trapping column (ZORBAX 300SB-C18, dimensions 5×0.3 mm 5μ m) and washed at a flow rate of 15 μ L/min for 5 minutes with a mobile phase of water containing 5% CH₃CN and 0.1% HCOOH. The trapping column was then placed in-line with the nano-pump at a flow rate of 350 nL/min. Water, with an 85 minute gradient from 8% CH₃CN to 40% CH₃CN was used to separate the peptides. The column was made from an in-house pulled 360/100 nm (outer/inner diameter) fused silica capillary packed with Jupiter C18 resin (Phenomenex; Torrance, CA). Data acquisition was performed using Xcalibur (version 2.0.6) software. The LC runs were monitored in positive ion mode by sequentially recording survey MS scans (m/z 400–2000) in the ICR cell, and $3 \times MS^2$ scan in the ion trap of the most intense ions. After two acquisitions of a given ion within a 45 seconds window, the ion was excluded from MS/MS analysis for a further 150 seconds [45,46].

MS Data Analysis

Default settings of the Raw Distiller program (UCSF) were used to create de-isotoped centroided peak lists from the raw spectra in the Mascot (x.mgf) format. These peak lists were searched against the NCBIInr (20070830; 5,422,622 entries) database with Viridiplantae (Green Plants) taxonomy restriction (Mascot™ server, Version 2.2, Matrix Science). Precursor mass tolerance was set to ± 6 ppm ; fragment ion tolerances were ± 0.8 Da. Trypsin specificity was used allowing for 2 missed cleavage. Met and Pro oxidation

were allowed for and Cys carbamidomethylation was set as a fixed modification. Mascot reporting was filtered to only allow for peptide matches with an ion score of > 6 and a protein hit requiring a bold red match. The expect cut off used for protein identification was $E < 0.005$ [45,46].

Results

When crude peanut extract (CPE) was passed over a gel filtration column (Fig. 1) the recovery of both protein ($75 \pm 7\%$) and effector activity ($76 \pm 16\%$) were similar (Table 2). The data in Fig. 2 correspond to those in Table 2 and demonstrate that fraction D (Fig. 1), with a theoretical average molecular weight of 20 kDa, which from this point on we will refer to as the 20 kD fraction (open circles), is more potent than either the CPE (closed squares), or any other fraction (other open symbols). As shown in Table 3, $85 \pm 2\%$ of the recovered activity was found in this fraction when fractions from the preparative column were assayed with IgE from the serum pool. Similar results were obtained with fractions from the analytical column assayed with the serum pool and from either column assayed with individual sera (Table 3). None of the other fractions consistently contained $> 5\%$ of the recovered activity (data not shown).

To test the hypothesis that the 20 kD fraction contains the majority of the effector activity in our CPE, all fractions were recombined with or without the 20 kD fraction and compared to the original CPE (Fig. 3). Proportional recombination of all fractions (closed circles) reconstituted the activity found in the CPE (closed squares). Two separate assays with the pool of 11 sera demonstrated that $140 \pm 43\%$ of the activity of the original CPE was found in the reconstituted material ($n=2$; Fig. 3A and data not shown). However, if all fractions except the 20 kD fraction were reconstituted, the 20 kD-depleted reconstituted material (open circles), had $8 \pm 2\%$ when assayed with the serum pool ($n=2$; Fig. 3A and data not shown) and $10 \pm 4\%$ when assayed with individual sera ($n=4$; Fig. 3 B–D and data not shown) of the activity compared to the CPE.

Figure 4 shows the molecular mass distributions of the CPE and the separate fractions collected following gel filtration. Samples were stained by silver stain (Fig. 4A) and by IgE immunoblot with a serum pool (Fig. 4B and 4C). Fraction D shows faint bands of 17 and 19 kD, presumably Ara h 2. Fraction E, the second most potent fraction (Table 2 and Fig. 2), contains a similar distribution of molecular masses to those seen in fraction D but the components are less abundant. Fractions F and G contained a variety of very faint bands (difficult to visualize in this exposure) that are likely the result of non-specific absorption of peanut proteins to the column.

Proteins in the 20 kD fraction were identified by two independent analytical strategies. First, the intact proteins ($800 \mu\text{g}$) were separated by two dimensional gel-electrophoresis (2D-GE) and stained. The protein spots were excised and digested with trypsin. Mass fingerprinting was performed by MALDI-TOF mass spectrometry. Almost all of the protein spots were identified as variants of Ara h 2 and h 6 (Figure 5, lower image and Table 4, column 7). The various forms of Ara h 2 were grouped into 3 categories: variants of 2.0101 (spots #1–10), variants of 2.0201 (spots #11–23) and those that were indistinguishable (i.e. forms of Ara h 2 where only shared peptides were identified; spots #24–27). We found no evidence for the presence of the Ara h 2 isoforms identified in the literature as 2.0102 and 2.0202. We also identified Ara h 6 (spots #28–45) but could not distinguish between 2 of the possible isoforms because the spots only contain peptides that are common to both forms (Table 4). A few spots (#24–27) yielded only peptides that were common to Ara h 2 and Ara h 6 and so their absolute identity could not be determined. Based on staining intensity, we estimate

that the isoforms of Ara h 2 and Ara h6 represent greater than 97% of the total protein in this fraction.

IgE immunoblots were also performed on separate 2D gels to further characterize the contents of the 20 kD fraction (Figure 5, upper right). To avoid smearing, only 25 µg of the 20 kD fraction was run. Spots corresponding to spots 1, 2, 4 (Ara h 2.01) and spots 12, 13, and 14 (Ara h 2) had the strongest immunoreactivity.

A second sample of the same fraction was digested with trypsin and the mixture of proteolytic peptides was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a high resolution LTQ-ICR mass spectrometer. Consistent with the results obtained by 2DE and peptide mass mapping, isoforms of Ara h 2 and h 6 were identified as the major components of the fraction. Table 4 reports the sequence coverage, protein identification score and includes the exponentially modified protein abundance index (emPAI) values as a measure of relative abundances. The emPAI value indicates that greater than 97% of the identified proteins in this fraction are variants of Ara h 2 and 6. By LC-MS/MS, the majority of the tryptic peptides can not unambiguously be assigned to a simple protein forms and therefore the relative abundances of each variant are inaccessible by this approach. However, several isoform-specific tryptic peptides were identified including three that distinguish the truncated version of Ara h 2 (2.0101 vs. 2.0201) and two forms of Ara h 6. Because the multiple protein isoforms were not separated prior to digestion and LC-MS/MS analysis, the peptides we identified for Ara h 2 can only be mapped to the specific isoforms listed in the protein sequence database (2.0101 and 2.0201). Another remarkable finding is that variants of Ara h 2 and Ara h 6 have highly variable isoelectric points suggesting extensive posttranslational modifications.

Discussion

In these studies we find that the majority (80–90%) of the effector activity of a CPE recovered from a gel filtration column resides in a single fraction comprised of components in the 13–25 kD range that we refer to as the 20 kD fraction.

Our analysis of this fraction by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI MALDI-TOFMS) and automated tandem mass spectrometry coupled to online capillary liquid chromatography (LC-MS/MS) identified two isoforms of Ara h 2, two isoforms of Ara h 6, multiple post-translationally modified variants of these proteins, and small amounts of other known allergens, Ara h 7 and Ara h 8. The few other proteins shown in Table 4 are of uncertain significance. We estimate that >97% of the protein mass in the biologically active 20 kD fraction consists of Ara h 2, Ara h 6, and their variants (Figure 5). The proteins that, on immunoblot, most strongly bind IgE from peanut allergic subjects are Ara h 2.0101 and Ara h 2.0201 (Figure 5, insert). In previous studies we have shown that selective removal of ~99 % of Ara h 2 as assessed by 2D gel and immunoblots resulted in a very small decrement in effector activity [41]. Koppelman and colleagues have demonstrated that Ara h 6 and Ara h 2 are approximately equipotent as measured by skin testing and *ex vivo* basophil activation [47]. Taking the current findings and this previous work together, we conclude that Ara h 2, Ara h 6, and their variants account for the majority of the effector allergic activity in peanuts. An important further conclusion from these observations is that, although Ara h 1 and Ara h 3 are reported to be major peanut allergens based on their ability to bind IgE, they do not co-purify with the majority of the effector activity found in a crude peanut extract and, in their native state, are not effective cross-linkers of IgE.

The ability of an allergen to effectively cross-link IgE and activate mast cells and basophils is referred to as its effector activity. By contrast, allergenicity is most commonly used to define an allergen's ability to elicit an IgE response, but it is also sometimes used to refer to the ability of an allergen to elicit an IgE response, the capacity to bind IgE, and/or to effect an IgE-mediated response. Arguably, the allergens with the greatest effector activity are the most clinically important. The effector activity of allergens depends upon: the number of epitopes, the affinity of those epitopes for IgE, the stereo-orientation of the epitopes, the concentration and the frequency of the IgE directed against those epitopes, and the molar amount of the protein bearing those epitopes [48–51]. The allergenicity of a protein is therefore not an accurate measure of its effector activity. The RBL SX-38 cells used in this study are an excellent model system for evaluating the effector activity of allergens. This is a stable cell line that effectively binds human IgE and is easy to activate. Data with this cell line are similar to, but more reproducible than those obtained with basophil histamine release assays [41].

We acknowledge that this study has some limitations. First, all of the sera used are from patients with very high levels of anti-peanut IgE. We did this to ensure sufficient sensitivity and a good signal-to-noise response, even in the presence of small concentrations of peanut allergens. Thus, different results may be found in patients with lower amounts of anti-peanut IgE. Second, restrictions in the US require that skin test reagents have investigational new drug (IND) approval. Consequently, we were not able to perform skin test titrations to independently verify these findings *in vivo*. This would be a useful adjunct to these findings. Third, the fractions from the gel filtration accounted for $76 \pm 16\%$ of the activity in our original CPE and 85% of this activity is in the 20kD fraction. So we can say that at least ~63% of the original effector activity is in the 20 kD fraction. We have not accounted for the ~24% of the activity that was not recovered from the column although, it is most likely that this loss was non-specific. Finally, only soluble native proteins were examined and additional epitopes, potentially from insoluble proteins or generated from proteins of larger molecular mass such as Ara h 1 and Ara h 3, following digestion or during food processing may be clinically important.

In conclusion, we have shown that for IgE from highly peanut allergic subjects, the peanut proteins, Ara h 2.0101, 2.0201, Ara h 6 isoforms, and their variants account for the majority of the effector activity in a saline extract of raw Georgia Green peanuts. It remains to be seen if this applies to all species of peanuts, all preparations of peanuts, and to all patients with peanut allergy.

Acknowledgments

The authors thank Drs. Heather Thompson and Shahzad Mustafa for helpful advice. We also thank Drs. Alan Bock, Jerry Koepke, Jeffery Rumbyrt, Katherine VanKerkhove, Karen Andrews, Allen Addinoff, and Leon Greos for referring patients and our study subjects for their participation.

Supported by an NIH grant, AI052164-01 to Dr. Dreskin, NIH National Center for Research Resources grant M01 RR000051 to the University of Colorado at Denver, and institutional funds.

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Figure 1.

A typical chromatogram following gel filtration (HiLoad 26/60 Superdex 75 Prep-Grade column; column volume ~325 ml) of CPE. Ten ml of clarified CPE (33 mg/ml) was applied and run at 2 ml/min. Absorption at 280 nm is shown on the vertical axis (mAU: milli Absorption Units). The 20 kD fraction is similarly resolved on Superdex 200 and on Superdex 75 of large and small configurations. Fractions and theoretical MW: A, ≥ 250 kD; B, 145kD; C, 100kD; D, 20kD; E, 7kD; F, 1kD; G, 0.7kD. Fractions F & G contain proteins non-specifically retained by the matrix (see Fig. 4). No proteins were detected in fractions H & I based on silver staining of a1D gel.

Figure 2.

Dose response of activity vs. protein concentration for fractions collected from the column (Fig. 1). All values are expressed as a % of maximal degranulation seen with 250 ng/ml of CPE within each assay. All assays were performed in triplicate and data points are mean $\pm$ SD. If standard error bars are not visible, the error was smaller than the symbol. Fig. 2A, assayed with a pool of 11 sera. Fig. 2B–D, assayed with 3 individual sera (B, D19; C, D60; and D, D62). Data are shown for the CPE (■, solid lines) and for fractions A–E (Open symbols with dashed lines: A, Δ ; B, ∇ ; C, $\diamond$; D, $\circ$; E, $\square$). Fractions F–K (data not shown) contained less effector activity than other fractions (see Table 2) and are not shown. The small peak between fractions C and D displayed no measurable activity (data not shown). Similar data were obtained with fractions from the analytic column with 4 individual sera (3 different from those shown) and with a pool of 10 sera (8 sera different from the serum pool shown).

Figure 3.

Dose response of activity vs. protein concentration for CPE (■, solid lines), all fractions proportionally recombined (●, dashed line), and all fractions except for the 20 kDa fraction proportionally recombined (⊗, dashed lines). Fig. 3A, assayed with a pool of 10 sera: Fig. 3 B–D, assayed with 3 individual sera (B, D19; C, D60; and D, D61).

Figure 4.

1D gels (15 % PAGE) and immunoblots (pool of 10 sera) for the CPE and the fractions A–G. (See Fig. 1.) Lanes (each with 3 μ g of protein): CPE and fractions A, B, C, D, E, F, and G as marked. A, silver stain; B & C, IgE immunoblot with the pool of 11 sera diluted 1:5 to give anti-peanut IgE = 18 U/ml. The exposure for B was for 1 minute, 12 minutes after adding the ECL reagent and for C, the exposure was for 15 seconds, 17 minutes after adding the ECL reagent.

Figure 5.

2D gel and IgE immunoblot of fraction D (20 kD fraction). The polyacrylamide 2D gels were pH 4–10 IEF and 4–16% gradient. For the image shown in the upper left and the lower image, 800 μ g of the 20 kD peak was loaded onto the gel and it was stained with lava purple and scanned prior to picking spots for Mass Spec analysis as described in the methods section. For the image shown in the upper right, 50 μ g of the 20 kD peak was loaded onto the gel. After transfer to PVDF membrane the sample was probed with a pool of 8 sera (a subset of the 11 sera used for the functional assays); diluted 1:10 to give anti-peanut IgE = 9.5 U/ml. Exposure for the immunoblot was for 15 seconds, 10 minutes after adding the ECL reagent. Attempts to perform immunoblots on membranes with larger amounts of protein led to smearing and high background. Spots #1–10, Ara h 2.0101. Spots #11–23, Ara h 2.0201. Spots # 24–27, could not distinguish between Ara h 2 and Ara h 6. Spots #28–45, Ara h 6. Spots #46–48, Ara h 7. Spot #49, Cyclophilin. Spot #50, Late embryogenesis abundant (LEA) protein (*Gossypium hirsutum*), a contaminant.

Table 1

Characteristics of the patients and their sera

Subject	Symptoms	IgE (IU/ml)	Anti- Peanut IgE kIU/L)
Serum Pool*		507	94
D18	Resp./Hives	161	64
D19 ^{*†}	Resp./Hives	124	50
D26	Syst. Anaph.	373	90
D29	Hives	106	25
D60 ^{*†}	Resp./Hives/ AE	295	50
D61 ^{*†}	Resp./Hives/ LOC	454	159
D62 ^{*†}	Hives/AE	161	64
D63 ^{*†}	Hives/AE	186	77
D64 [*]	Resp./GI	56	21
D66 [*]	Hives/AE	2416	113
D69 ^{*†}	Hives/AE/GI	568	137
D70 ^{*†}	Resp./GI/AE/ BP	245	53
D71 ^{*†}	Resp./Hives/ AE/BP	560	216
D73 [*]	Hives	697	334

* These 11 sera were pooled for the functional assays shown in Fig. 2 and 3 and for the immunoblot shown in Fig. 4B and C (Geom mean: total IgE = 320; Peanut-specific IgE = 88).

[†] These 8 sera (Geom means: total IgE = 374; Peanut-specific IgE = 95) were used for the immunoblot shown in Fig. 5. Sera were pooled so that each serum sample contributed the same amount of anti-peanut IgE based on the ImmunoCap® data. The IgE values for the serum pool are therefore not simply the arithmetic mean for the individual sera samples. Symptoms are those associated with each subject's worse reaction: Resp., wheezing; Anaph., anaphylaxis; GI, gastrointestinal symptoms; AE, angioedema; LOC, loss of consciousness; BP, blood pressure (hypotension).

Table 2

Effector activity of CPE and fractions from gel filtration

Fraction	Approx average MW (range)	Protein n=3 runs	Separate chromatographic runs*		
			#1	#2	#3
	kD	mg	Relative units ($\times 10^{-5}$)		
CPE	NA	337 $\pm$ 44	130	228	16
A	342 (167–342)	105 $\pm$ 3	6.7	10	0
B	144 (117–167)	15 $\pm$ 1	0.7	0.7	0
C	99 (58–117)	36 $\pm$ 3	2.8	2.7	0
D	18 (13–25)	28 $\pm$ 2	108	175	7.2
E	7 (4–10)	14 $\pm$ 0	9.8	2.3	0
F	0.7 (0.3–1)	36 $\pm$ 24	2	0.3	0
G	<1	17 $\pm$ 10	0.5	0.5	0
>G	<1	5 $\pm$ 1	0	0	0
% Recovery (activity)			100%	84%	45%
% Recovery (activity) mean $\pm$ SEM			76 $\pm$ 16%		
% Recovery (protein) mean $\pm$ SEM		75 $\pm$ 7%			

NA, not applicable.

* Samples from separate chromatographic runs of a preparative preparative Superdex 75 column (Fig. 1). A fourth run on an analytical column gave similar data (recovery of protein = 75%; recovery of activity = 67%).

Table 3

Proportion of the total activity found in the 20 kD fraction with different columns and multiple sera.

Column	Cells sensitized with:	Recovered activity in 20 kD fraction (% of total recovered)
Preparative Superdex 75	Serum D19	79
	Serum D60	88
	Serum D62	81
	Mean $\pm$ SD measured with each of 3 sera	83 $\pm$ 5
	Mean $\pm$ SD measured with serum pool (n=3)	85 $\pm$ 2
Analytical Superdex 75	D18	81
	D19	58
	D26	81
	D29	98
	Mean $\pm$ SD measured with each of 4 sera	80 $\pm$ 16
	Mean $\pm$ SD measured with a single serum	89

Protein Identification

Table 4

Accession #	Protein * Name	Theoretical MW	Search Score	Seq. Coverage	emPAI	MFP ID Top score	Seq. Coverage
gi 148613175	Ara h 2.0101	20907	7907	73	853.3	192	69
gi 31322017	Ara h 2.0201	20212	6596	69	219.8	191	79
gi 75114094	Ara h 6 isoform	17479	4325	64	122.2	176	74
gi 17225991	Ara h 6 isoform	17349	4278	64	125.9	168	75
gi 145904610	Ara h 8	16208	1736	42	24.1	81	63
gi 66735602	LEA protein	10408	672	42	4.1	80	45
gi 115187464	thioredoxin fold	17525	480	20	0.5		
gi 15226956	osmotin-like ¹	27912	346	8	0.5		
gi 18499	LEA protein ²	11965	259	35	0.8		
gi 83776794	LEA 4	5802	267	42	2.0		
gi 158121995	Ara h 7					130	55
gi 3334157	Cyclophilin					92	43

* All matches to *Arachis hypogaea* protein sequences unless stated otherwise

¹ *Arabidopsis thaliana*

² Late embryogenesis abundant (LEA) protein (*Gossypium hirsutum*)