Ara h 6 Complements Ara h 2 as an Important Marker for IgE Reactivity to Peanut

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INTRODUCTION

Peanut allergy is an increasingly important food allergy, with high prevalences and severe clinical symptoms (1). Ara h 1 and Ara h 2 have been well-established as the major peanut allergens. However, newer studies have found that Ara h 6 has a similar seroprevalence as Ara h 2 and may play a more significant role in peanut allergy than previously thought (2). Ara h 6 and Ara h 2 also share similarities in molecular weight (Ara h 2: 17-18 kDa, Ara h 6: 14.5 kDa) and amino acid sequence with 55% identity*. This homology provides a challenge for obtaining natural Ara h 6 that is free of Ara h 2. Our study compares the IgE antibody activity of highly purified natural Ara h 6 (nAra h 6) to a natural Ara h 6 reference material, recombinant Ara h 6 (rAra h 6) and natural Ara h 2 (nAra h 2). * Calculated based on the sequence of Ara h 2.0101, accession no. AY007229 and Ara h 6.0101, AF092846.

METHODS

Purified nAra h 6 was analyzed by silver-stained SDS-PAGE and tandem mass spectrometry (LC/MS-MS). Ara h 2 level in the purified material was analyzed by monoclonal antibody ELISA. An Immunoblot was also performed on nAra h 6, nAra h 2, and a peanut extract with a serum pool of 6 peanut allergic patients. Increasing concentrations of nAra h 2 or nAra h 6 were incubated with sera prior to analysis by chimeric-IgE ELISA in a cross-inhibition test. Specific IgE levels to nAra h 6, nAra h 2, the reference material and rAra h 6 were also compared by chimeric-IgE ELISA. The biological activities of nAra h 6 and nAra h 2 were compared in a stripped basophil histamine release assay.

RESULTS

Purification of nAra h 6 and IgE reactivity in Immunoblot: Highly purified natural Ara h 6 contained 0.005% of Ara h 2 as measured by monoclonal antibody ELISA (data not shown). SDS-PAGE of the reference material (Fig. 1, lane 1) and the purified nAra h 6 (Fig. 1, lane 2) showed a major band at the expected MW of 14.5 kDa. Natural Ara h 2 (Fig. 2, lanes 1, 2), peanut extract (Fig. 2, lane 3) and nAra h 6 (Fig. 2, lanes 4, 5) were blotted using a pool of peanut allergic patients' sera.

Fig. 1

Fig. 2

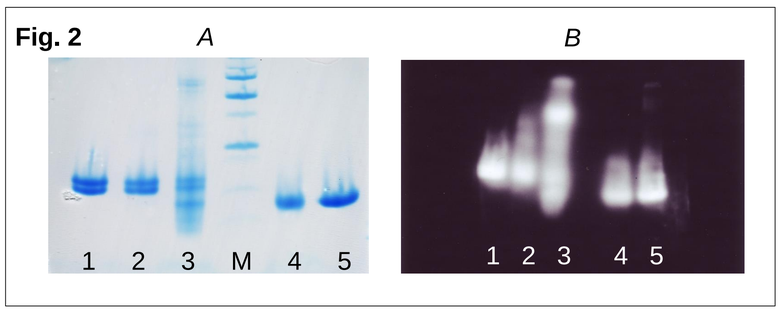

Fig. 1: SDS-PAGE of purified natural Ara h 6 followed by silver staining. **Fig. 2:** ***A)*** SDS-PAGE of purified natural Ara h 2, Ara h 6, and peanut extract followed by Coomassie blue staining. ***B)*** Immunoblot of Ara h 2, Ara h 6, and peanut extract using a serum pool of 6 peanut allergic patients. Proteins transferred onto a PVDF membrane were probed with sera, and bound IgE was detected with a peroxidase-labeled goat anti-human IgE.

Cross-inhibition test: Due to the homology of Ara h 2 and Ara h 6, cross-inhibition assays were performed by chimeric-IgE ELISA on a serum pool pre-incubated with Ara h 2 or Ara h 6. At 70 ng/ml, Ara h 2 inhibited 50% of IgE-Ara h 2 and IgE-Ara h 6 interactions (Fig. 3, red line). Much higher concentrations – 600 ng/ml and 5000 ng/ml – of Ara h 6 were required to inhibit 50% of IgE binding to Ara h 6 and to Ara h 2 respectively (Fig. 3, yellow line).

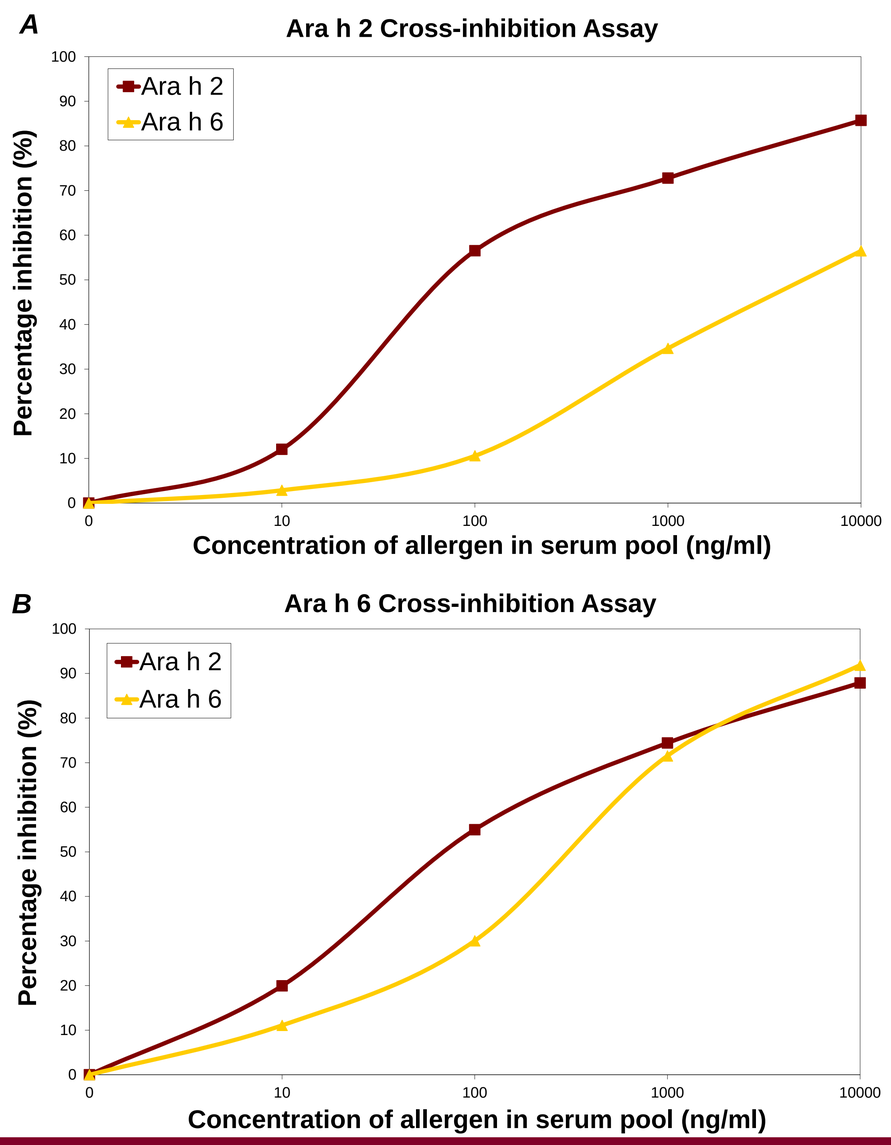

Fig. 3 Specific IgE cross-inhibition assay with increasing concentrations of Ara h 2 and 6. **A)** Percent inhibition of Ara h 2-specific IgE binding activity. **B)** Percent inhibition of Ara h 6-specific IgE binding activity.

Biological activity of Ara h 6: A basophil histamine release assay was performed using sera from 20 peanut allergic patients. Ara h 2 was more potent, reaching 50% of maximum histamine release at 0.05 ng/ml compared to Ara h 6 at 1 ng/ml (Fig. 4).

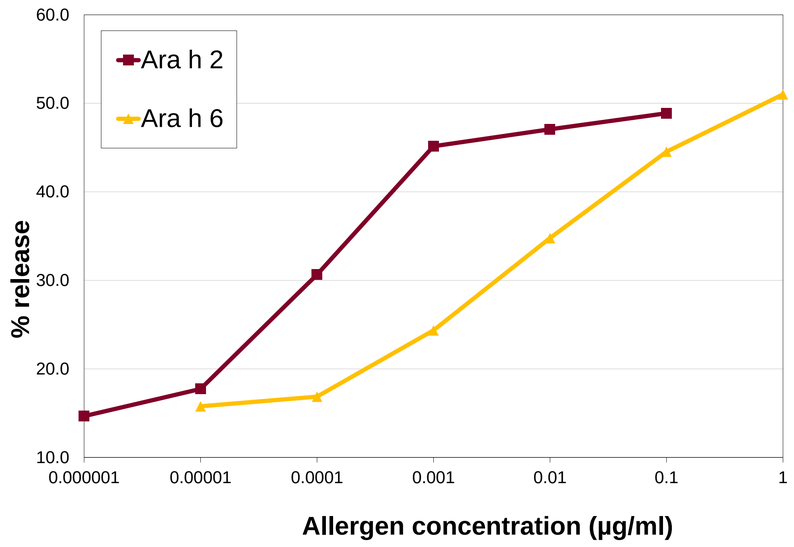

Fig. 4 Average basophil histamine release by nAra h 2 and nAra h 6 (n = 20) with increasing concentrations of allergen.

IgE reactivity of Ara h 6: 45 out of 54 sera from peanut allergic patients (83%) tested using chimeric-IgE ELISA showed IgE reactivity to nAra h 6 compared to 39 of the sera (72%) reacting to nAra h 2, thus supporting the importance of Ara h 6 in studying and diagnosing peanut allergy (2). IgE binding to nAra h 6 correlated well with IgE binding to the reference material (Fig. 5A) and nAra h 2 (Fig. 5B). IgE binding to recombinant Ara h 6 also correlated well to the purified natural allergen. However, the IgE activity level was generally higher for the highly purified nAra h 6 (data not shown).

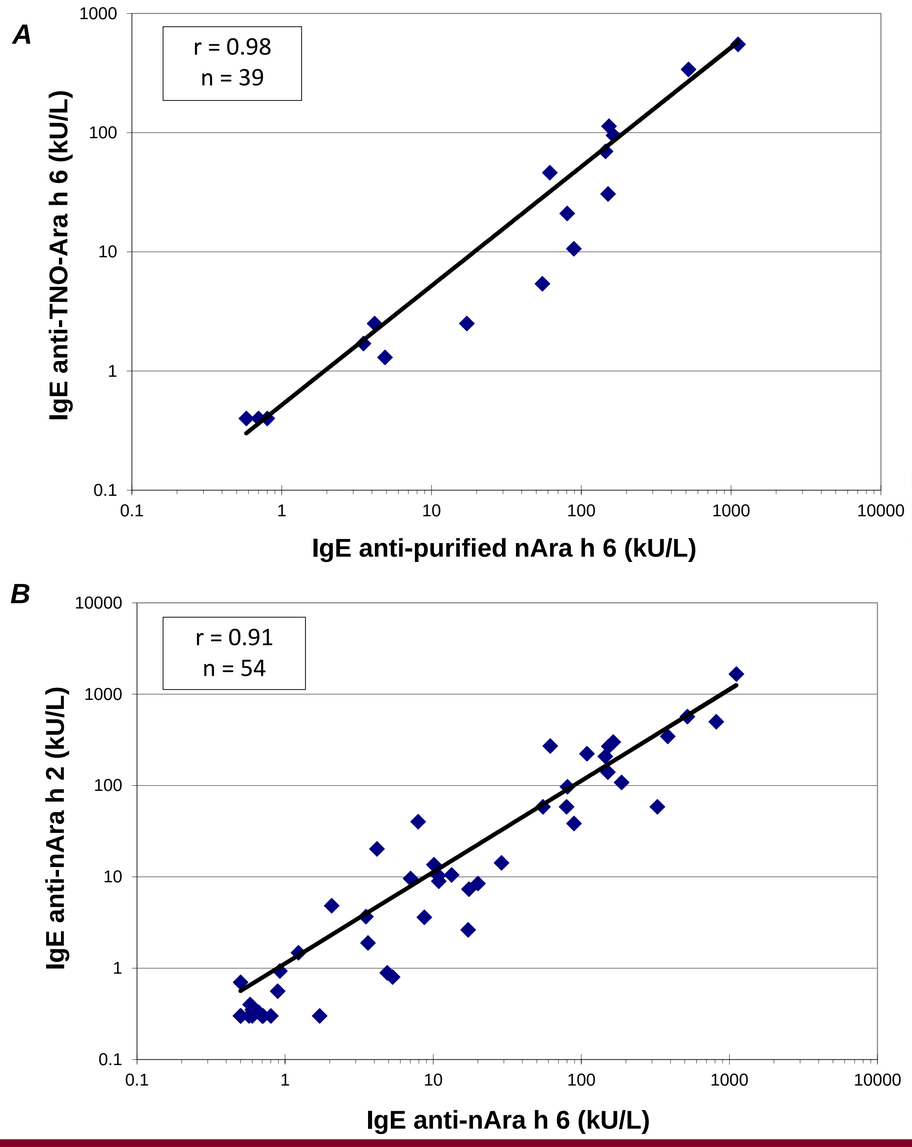

Fig. 5: Correlation of IgE binding to nAra h 6 and to the reference material **(A)** and to nAra h 2 **(B)**.

CONCLUSIONS

Purified nAra h 6 was recognized by more sera from peanut allergic patients than nAra h 2 (83% and 72% respectively). However, nAra h 6 was less potent since higher concentrations were required to elicit the same level of response as nAra h 2 in cross-inhibition and basophil histamine release assays. Together with purified nAra h 2, the highly purified Ara h 6 will be useful for diagnostic IgE antibody assays and other molecular and cellular studies to further investigate the immunological mechanism of peanut allergy.

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Ara h 6 Complements Ara h 2 as an Important Marker for IgE Reactivity to Peanut

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ABSTRACT: The similarities of two major peanut allergens, Ara h 2 and Ara h 6, in molecular size, amino acid sequence, and structure have made it difficult to obtain natural Ara h 6 free of Ara h 2. The objectives of this study were to purify natural Ara h 6 that is essentially free of Ara h 2 and to compare its IgE reactivity and potency in histamine release assays to Ara h 2. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis of the highly purified allergen (<0.01% Ara h 2) revealed a single 14.5 kD band, and the identity of Ara h 6 was confirmed by liquid chromatography−tandem mass spectrometry. Ara h 6 showed a higher seroprevalence in chimeric IgE enzyme-linked immunosorbent assay (n = 54) but a weaker biological activity in basophil histamine release assays than Ara h 2. Purified Ara h 6 will be useful for diagnostic IgE antibody assays as well as molecular and cellular studies to investigate the immunological mechanisms of peanut allergy.

KEYWORDS: *peanut, allergen, IgE reactivity, histamine release*

INTRODUCTION

Peanut allergy is an important food allergy in the United States that affects 1.4% of children and 0.6% of adults.[1,2] In the United Kingdom, approximately 1.8% of children have an allergy to peanut.[3,4] While most children outgrow food allergies to milk, egg, or wheat, allergy to peanut is more persistent and often continues into adulthood.[5] Minute amounts of peanut, as little as 0.4 g, are enough to elicit milder allergic symptoms that include rashes, angioedema, and gastrointestinal symptoms.[5,6] However, peanut is also one of the main triggers of severe anaphylactic reactions that can be fatal.[7]

Currently, 12 peanut allergens have been documented by the World Health Organization (WHO)/International Union of Immunological Societies (IUIS) Allergen Nomenclature committee.[8] Ara h 1 and Ara h 2 have been well-studied and are recognized as major allergens. Ara h 2 has a higher predictive value for diagnosis of clinical peanut allergy than Ara h 1, Ara h 3, Ara h 8, and Ara h 9.[9] Ara h 2 is also more potent than Ara h 1 or Ara h 3 in histamine release assays and skin prick tests.[10−12]

Another peanut allergen, Ara h 6, has recently emerged as an important allergen, which, together with Ara h 2, has been associated with clinical peanut allergy.[13] Ara h 6 has approximately the same seroprevalence as Ara h 2 and thus is considered a major peanut allergen.[13,14] Ara h 6 and Ara h 2 are of similar molecular size; Ara h 2 is 17−19 kDa, and Ara h 6 is 14.5 kDa. Both allergens are 2S albumins that are heat-stable, immunogenic, and resistant to digestion in the gut.[15−17] The nucleotide (and amino acid) sequences of Ara h 2.0101 and Ara h 6.0101 are 50% identical and 58% similar (EMBOSS Needle alignment). The structure of the protease-resistant core of Ara h 6 has previously been determined by nuclear magnetic resonance (NMR), and the folds of this allergenic core were found to be virtually identical to those of Ara h 2.[15] The three-dimensional folds determine the IgE epitopes, which are lost upon unfolding.[18] More recently, the structure of Ara h 2 was determined by X-ray crystallography, and molecular modeling studies predict that half of the residues on the surfaces of both proteins are conserved.[19] These factors contribute to the difficulty in obtaining purified natural Ara h 6 (nAra h 6) that is free of Ara h 2. Ara h 7, the third peanut allergen of the 2S albumin family, also shows sequence homology to Ara h 2 and Ara h 6.[20] However, attempts to identify natural Ara h 7 protein in peanut extracts have failed. Recently, Schmidt et al. found low abundance of natural Ara h 7.0201 and Ara h 7.0202 in peanut extracts after enrichment of the low-molecular-mass peanut proteins.[21]

Highly purified allergens are important for allergen standardization.[22,23] There is extensive variability in allergen composition and potency between different commercially available peanut extracts, including those used for skin prick testing that could lead to misdiagnosis.[24,25] "Component-resolved" or molecular diagnostics is based on measuring IgE antibodies to multiple individual allergens rather than to heterogeneous extracts. The use of molecular diagnostics tends to lower false-positive IgE antibody results caused by the interaction with profilins and cross-reactive carbohydrate

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determinants that are present in diverse plant-based food.[26−29] Some birch pollen-allergic patients were found to be cross-sensitized to peanut through cross-reactivity between Bet v 1 and Ara h 8, which are homologous proteins that belong to the PR-10 family.[30] Moreover, pea-allergic patients, who later developed peanut allergy, recognized only Ara h 1 but not Ara h 2 or Ara h 3.[31] Vicilin homologues in pea and peanut (Ara h 1) are the molecular basis for this cross-reactivity. Most peanut-allergic patients that react to Ara h 2 also have IgE against Ara h 6; however, a recent case study reported that Ara h 6 caused severe reactions to peanut in the absence of sensitization to Ara h 2.[32] Thus, it is important to have pure, single-component peanut allergens for accurate diagnosis of peanut allergy.

Our objective in this study was to obtain purified nAra h 6 that is essentially free of Ara h 2 and compare its IgE reactivity and potency in histamine release assays to Ara h 2.

MATERIALS AND METHODS

Purified Natural Ara h 6. Peanut flour was extracted into phosphate-buffered saline (PBS) at pH 7.4 containing 1 M sodium chloride for 2 h at 60 °C. The mixture was centrifuged at 13500*g* for 25 min, and the supernatant was sterile-filtered. Ammonium sulfate precipitation was used to remove contaminating Ara h 1. The 70−100% pellet containing Ara h 6 and Ara h 2 was redissolved in PBS and passed over a 1C4 monoclonal antibody column to remove the contaminating Ara h 2. Size-exclusion chromatography (HiPrep 16/60 Sephacryl S-100 HR, GE Life Sciences, Uppsala, Sweden) was used as a final polishing step. The identity of purified nAra h 6 was confirmed by liquid chromatography−tandem mass spectrometry (LC−MS/MS), and its purity was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and silver staining. The concentration of the purified protein was determined by the Advanced Protein Assay (Cytoskeleton, Denver, CO), and the Ara h 2 content was measured using a two-site antibody enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody 1C4 as the capture antibody and rabbit-polyclonal antibody anti-Ara h 2 for detection.[33] Ara h 6 was measured by ELISA using capture monoclonal antibody 3B8 and detection antibody 3E12. Another nAra h 6 preparation available from TNO Quality of Life, Zeist, Netherlands (T-Ara h 6) was tested for comparison.

Sera. Sera for the immunoblotting, chimeric IgE ELISA, and cross-inhibition ELISA were obtained from Bioreclamation, Inc. (East Meadow, NY) (n = 24), which operates in full compliance with the Food and Drug Administration guidelines, the Department of Allergy and Clinical Immunology at Johns Hopkins University (n = 25), and kindly provided by Dr. Peter Heymann, University of Virginia (n = 8). Sera for the Basophil Histamine Release Assay were provided by the Department of Allergy, Asthma, and Clinical Immunology at the University of Colorado (n = 20). All donors signed informed consent and, for minors, assent. Sera were collected with approval from the respective institutions' human investigation committees.

Immunoblotting. IgE reactivity of purified nAra h 6 was compared to natural Ara h 2 (nAra h 2), peanut extract, and T-Ara h 6 by immunoblot using pooled sera from six peanut-allergic patients (≥100 kU/L by chimeric IgE ELISA, PWH6, BR13, BR21, BR22, BR23, and BR24). Proteins loaded at 0.5 mg/mL and separated by SDS−PAGE under non-reducing conditions (20% homogenous, GE Lifesciences, Uppsala, Sweden) were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA) by electroblotting. The membrane was blocked with 5% non-fat dry milk in 25 mM Tris, 150 mM NaCl at pH 7.4, and 0.05% Tween-20 for 16 h at 4 °C and then incubated with the pooled sera at a 1:2 dilution in blocking buffer for 2 h at room temperature. Bound IgE was detected with peroxidase-labeled goat anti-human IgE (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) followed by ECL Western Blotting Analysis System (GE Lifesciences, Uppsala, Sweden), which produced a detectable chemiluminescent signal.

Chimeric IgE ELISA. IgE antibody binding to nAra h 6 was compared to IgE binding to T-Ara h 6 and nAra h 2 by a modified chimeric IgE ELISA as described previously.[34−36] Briefly, microtiter plates coated to saturation with one allergen at 0.5 μg/well were incubated with sera from peanut-allergic patients (ImmunoCAP values ranged from 0.45 to >100 kUA/L, with an average of 15.2 ± 21.4 kUA/L; n = 54). Bound IgE antibody was detected using biotinylated goat anti-human IgE (Kierkegaard and Perry, Gaithersburg, MD), followed by streptavidin peroxidase. The intensity of the color developed by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was measured at 405 nm. A chimeric anti-Der p 2 antibody, 2B12-IgE, was used to establish a standard curve from 0.4 to 115 U/mL IgE to quantify the bound specific IgE as previously reported.[36] Ara h 6- and Ara h 2-specific IgE for each serum sample were extrapolated from the linear portion of the 2B12−IgE curve. Levels of specific IgE antibody reactive with nAra h 6 and nAra h 2 or T-Ara h 6 were plotted and modeled using linear regression. Pearson correlations were used to analyze the relationship between specific IgE to nAra h 6 and nAra h 2 or T-Ara h 6 in sera of peanut-allergic patients.

Cross-inhibition ELISA. The chimeric IgE ELISA was modified for cross-inhibition assays. Microtiter plates were saturated with nAra h 6 or nAra h 2 at 0.5 μg/well for 16 h at 4 °C.[34] A serum pool of the same sera used in the immunoblot was incubated with increasing concentrations of the inhibitor (either nAra h 6 or nAra h 2) for 1 h prior to performing the assays. The protein concentration of the inhibiting allergen was increased in 10-fold increments from 10 to 10 000 ng/mL. The pooled sera with or without the inhibitor were added to their respective wells and incubated at room temperature for 1 h. Bound IgE was detected as described for the chimeric IgE ELISA. The percent inhibition was calculated on the basis of the optical density at 405 nm. A 0% inhibition was assumed for the wells that had no inhibitor.

Basophil Histamine Release Assay. The stripped basophil histamine release assay was performed as previously described.[37] Briefly, basophil cells were stripped of membrane-bound IgE and sensitized with each serum from peanut-allergic patients (Ara h 6-specific IgE values ranged from 5 to 480 kUA/L, with an average of 87.8 ± 125 kUA/L; n = 20). The cells were then stimulated with increasing concentrations of allergen, and histamine release was measured by fluorometric analysis. Histamine release was expressed as a percentage of the total amount of histamine in the cells determined after lysis of the cells with perchloric acid (100% release). EC_{50} was the allergen concentration required to stimulate 50% of the maximum release. Histamine release dose response curves for nAra h 6 and nAra h 2 were generated for concentrations between 0.001 and 1000 ng/mL.

RESULTS

Protein Characterization. Purified nAra h 6 (lane 4) and T-Ara h 6 (lane 5) migrated as single bands on silver-stained SDS−PAGE at the predicted molecular weight of 14.5 kDa (Figure 1A). The Ara h 6 content was 1.05 mg/mL, while the

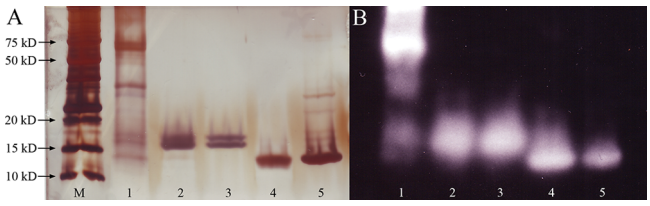

Figure 1. Silver stain and immunoblot of nAra h 2, nAra h 6, and peanut extract using pooled sera from six peanut-allergic patients. (A) Silver-stained gel. (B) Proteins transferred onto a PVDF membrane were probed with sera, and bound IgE was detected with a peroxidase-labeled goat anti-human IgE. Lane M, marker; lane 1, peanut extract; lanes 2 and 3, nAra h 2; lane 4, nAra h 6; and lane 5, T-Ara h 6.

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concentration of Ara h 2 was less than 0.1 μg/mL (0.01%) as measured by two-site antibody ELISA. The results of LC−MS/MS confirmed the identity of the purified allergen as Ara h 6.0101 with 74.5% sequence coverage (Figure 2).

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1    MAKSTILVAL LALVLVAHAS AMRRERGRQG DSSSCERQVD RVNLKPCEQH
51   IMQRIMGEQE QYDSYDIRST RSSDQQQRCC DELNEMENTQ RCMCEALQQI
101  MENQCDRLQD RQMVQQFKRE LMNLPQQCNF RAPQRCDLDV SGGRC
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Figure 2. Sequence coverage of purified nAra h 6 with Ara h 6.0101. Underlined red letters indicate identical amino acids between the purified allergen and the published sequence.

IgE Binding Analysis. Immunoblotting demonstrated strong IgE reactivity to nAra h 2 (lanes 2 and 3), nAra h 6 (lanes 4 and 5), and peanut extract (lane 1; Figure 1B). The major allergens, Ara h 1 (63 kDa), Ara h 2 (18−20 kDa), and Ara h 6 can be seen as major bands in the peanut extract. Ara h 2 was not detected in the purified nAra h 6 preparation.

More sera showed IgE binding to nAra h 6 than to nAra h 2 (45 versus 39) using a chimeric IgE ELISA; however, the levels of Ara h 2-specific IgE were on average 13% higher than the levels of Ara h 6-specific IgE. A total of 25 of these patients had higher levels of specific IgE to one allergen but not to the other (Table 1). For example, patient BR1 had 4-fold higher Ara h 2-specific IgE than Ara h 6-specific IgE, while patient BH25 had 5-fold higher Ara h 6-specific IgE than Ara h 2-specific IgE (Table 1). There was a strong quantitative correlation between the levels of specific IgE to nAra h 6 and specific IgE to nAra h 2 ($r = 0.91$; $n = 54$; $p < 0.001$; Figure 3A). The levels of Ara h 6-specific IgE binding to purified nAra h 6 correlated well with the same Ara h 6-specific IgE binding to T-Ara h 6 ($r = 0.98$; $n = 39$; $p < 0.001$; Figure 3B). On average, the IgE binding to the purified nAra h 6 material was 46% higher than that observed interacting with T-Ara h 6.

Results obtained in the cross-inhibition assay indicate that nAra h 2 inhibited IgE allergen binding more effectively than nAra h 6. At 70 ng/mL, Ara h 2 inhibited 50% of IgE−Ara h 2 and IgE−Ara h 6 interactions (Figure 4). However, 600 and 5000 ng/mL of Ara h 6 were required to inhibit 50% IgE binding to Ara h 6 and Ara h 2, respectively.

Biological Activity. Natural Ara h 6 showed less biological activity than nAra h 2 in the basophil histamine release assay (Figure 5). At a concentration of 0.1 μg/mL, Ara h 2 stimulated 50% of the maximum histamine release, while 1 μg/mL of Ara h 6 was required to achieve 50% of maximum histamine release. In general, histamine release was 10% lower for nAra h 6 than nAra h 2 at the same allergen concentration. The dose−response curve of histamine release by nAra h 6 was shifted to the right of the dose response curve for nAra h 2 (Figure 5). On average, histamine release began to plateau at 0.001 μg/mL Ara h 2 and continued an upward trend even to a concentration of 1 μg/mL Ara h 6.

DISCUSSION

Peanut allergy is on the rise, as evidenced by a 1% increase in just over an 11 year period (1997−2008) in the U.S.[2] Traces of peanut can be found in many processed foods, and this poses a danger to peanut-allergic patients because miniscule amounts can trigger an allergic reaction.[6] Previous studies have demonstrated significant variability in the protein content and allergenicity of commercially available peanut extracts.[38] There is a critical need for ultrapure allergens to clarify research results and improve the accuracy of diagnostic tests in an attempt to better understand the mechanisms associated with the induction of allergic diseases. Analysis of single components

Table 1. Ara h 2- and Ara h 6-Specific IgE Concentrations of Sera of Peanut-Allergic Patients That Were Positive to at Least One of the Two Allergens as Measured by Chimeric ELISA[a]

INDOOR ID	Ara h 2 (kUA/L)	nAra h 6 (kUA/L)	INDOOR ID	Ara h 2 (kUA/L)	nAra h 6 (kUA/L)
BH1	8.96	10.9	**BH25**	58.3	326
BH2	223	109	**BR1**	270	61.7
BH3	9.59	7.02	**BR2**	38.4	89.2
BH4	3.61	8.7	**BR4**	<0.4	0.7
BH5	8.43	20	**BR5**	20.2	4.17
BH6	345	383	BR6	3.67	3.51
BH7	499	814	**BR8**	0.89	4.89
BH8	1.03	10.8	**BR11**	<0.4	0.6
BH9	40.1	7.9	BR12	0.4	<0.4
BH10	0.56	0.89	BR13	568	520
BH11	<0.4	0.7	**BR14**	0	0.8
BH12	58.3	79.5	BR16	2.63	3.07
BH13	108	187	**BR17**	58.4	6.71
BH14	1.89	3.62	**BR18**	96.8	36.1
BH15	0.93	0.92	BR21	208	145
BH16	<0.4	1.71	BR22	140	151
BH17	13.6	10.1	BR23	299	164
BH18	1.05	13.3	**BR24**	269	93.1
BH19	7.32	17.4	PWH1	<0.4	0.57
BH21	0.35	0.6	**PWH3**	0.8	5.31
BH22	1.47	1.23	**PWH4**	0.33	0.66
BH23	14.2	28.9	**PWH5**	<0.4	0.71
BH24	4.83	2.06	PWH6	1657	1113

[a]Sera with a ratio of Ara h 2-specific IgE/Ara h 6-specific IgE of >2 or <0.5 are highlighted in bold font.

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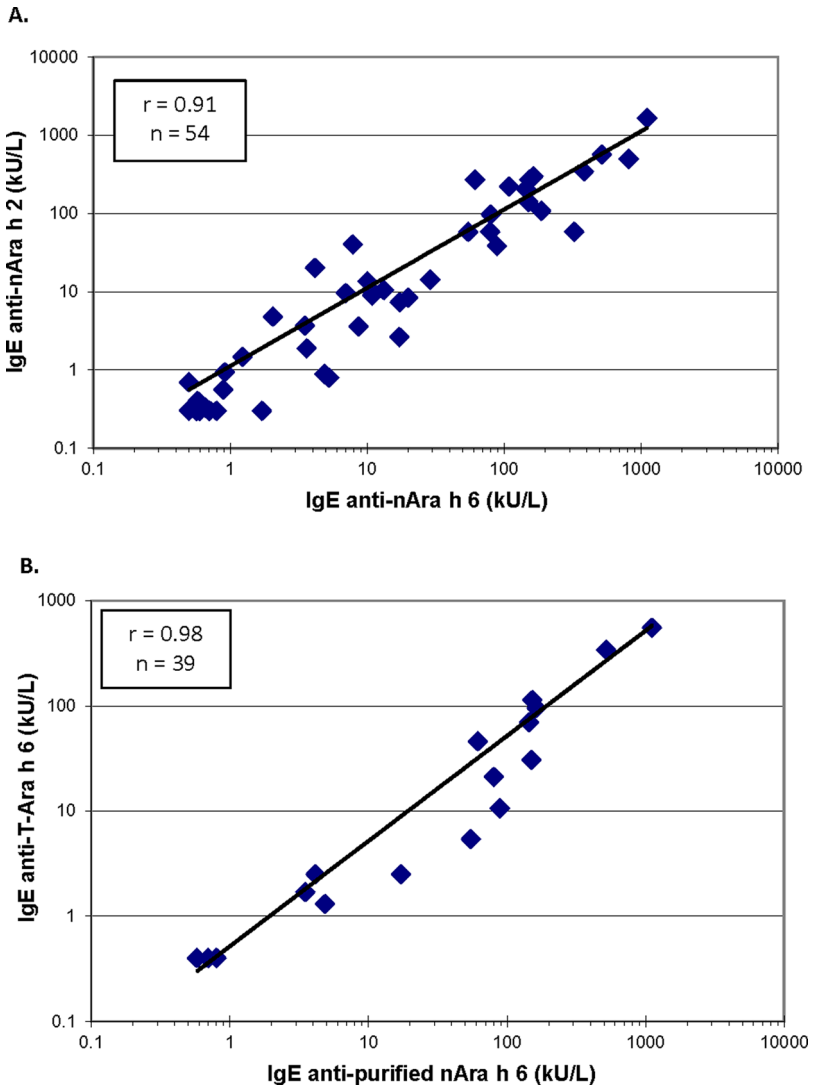

Figure 3. Correlation between (A) IgE binding to purified nAra h 6 and purified nAra h 2 and (B) purified nAra h 6 and T-Ara h 6. Allergen coated onto microtiter plates was incubated with sera from peanut-allergic patients, and bound IgE was detected by biotin-labeled goat anti-human IgE, followed by streptavidin peroxidase. The intensity of the color development of ABTS was measured at 405 nm.

allows for more effective dissection of cross-reactivity among structurally similar allergens and aids in identifying the cross-reactive allergen from the allergen that initially elicited the sensitization. For food allergy studies in particular, molecular diagnostics more effectively reveal patterns of sensitization and cross-reactivity among allergenic homologues across food groups.[39]

Ara h 2 has been extensively studied and established as a major allergen, while Ara h 6 has only recently gained interest in peanut allergy research. Ara h 2 and Ara h 6 belong to the 2S albumin family that are characterized by having a stable core formed by four disulfide bridges, which makes these proteins resistant to high-temperature processing and proteolytic digestion.[16,40] The stability of these proteins and their resistance to digestive enzymes in the gut has been identified as major factors contributing to their allergenicity.[15,41] The sequence and structural similarity between Ara h 6 and Ara h 2 has made it difficult to produce nAra h 6 free of nAra h 2.[19] Recombinant Ara h 6 has been produced, but conformational differences between the natural and recombinant forms have yielded recombinant Ara h 6 with low IgE reactivity.[20] More recently, a recombinant Ara h 6 produced in *Pichia pastoris* has shown improved biological potency similar to that of nAra h 6.[42]

Highly purified nAra h 6 has analytically been shown to be free of Ara h 2. Similar to natural Ara h 2, natural Ara h 7 also shares sequence similarity with Ara h 6 and thus may co-purify with Ara h 6. However, natural Ara h 7 has a very low abundance in peanut extracts, which greatly minimizes the risk of contamination.[21]

Purified natural Ara h 6 displayed its own unique immunological activity patterns. While a strong correlation was shown between the relative levels of Ara h 2-specific IgE and Ara h 6-specific IgE, sera from 25 individuals reacted more strongly to one allergen than the other, as reported in previous studies.[16,32,43] Interestingly, only 8 of the 25 tested sera had higher Ara h 2-specific IgE, even though Ara h 2 is the predominantly recognized allergen by peanut-allergic patients.[13]

In our cross-inhibition analyses, Ara h 2 inhibited Ara h 6-specific IgE binding more effectively than Ara h 6 inhibited Ara h 2-specific IgE binding as reported previously.[16] The inhibitory

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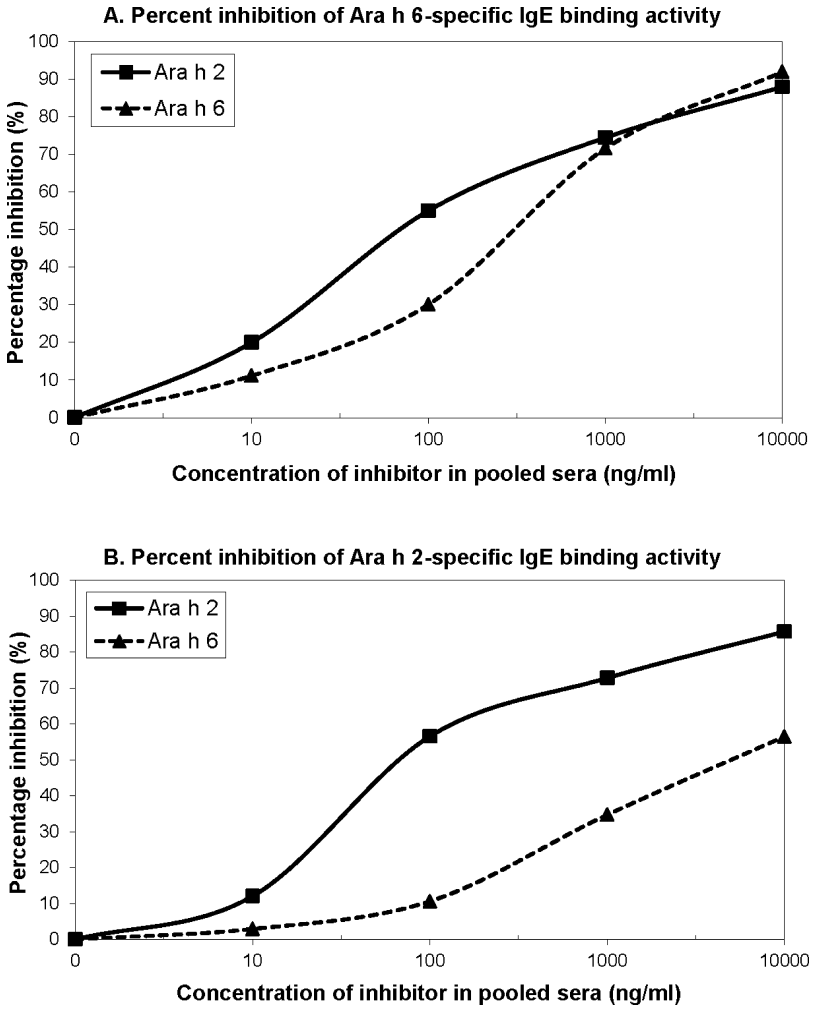

Figure 4. Specific IgE cross-inhibition assay with increasing inhibitor allergen concentrations of Ara h 2 and Ara h 6. (A) Percent inhibition of Ara h 6-specific IgE binding activity. (B) Percent inhibition of Ara h 2-specific IgE binding activity.

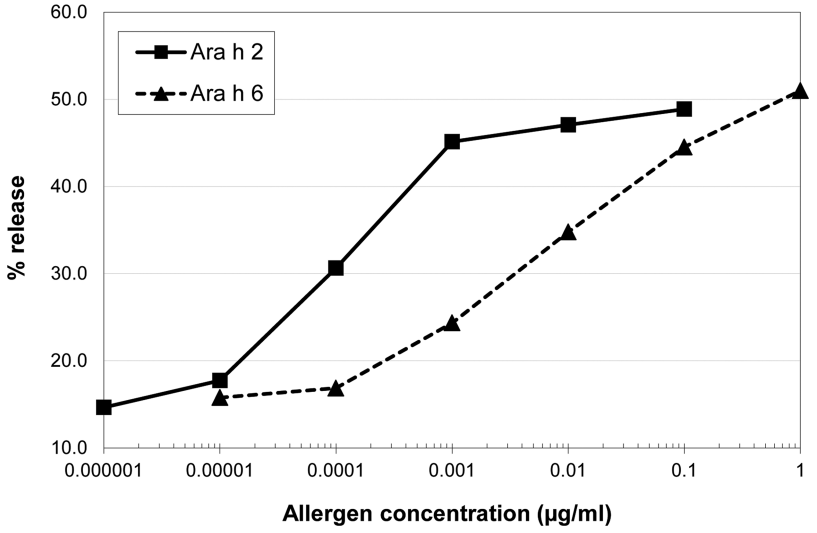

Figure 5. Average basophil histamine release by nAra h 2 and nAra h 6 (n = 20) using increasing concentrations of allergen.

effect of Ara h 2 may be due to its unique IgE-binding epitopes that are not present in Ara h 6, while Ara h 6 contains IgE-binding epitopes that are also present on Ara h 2.[44] However, we did not achieve complete inhibition of Ara h 6−IgE binding by Ara h 2 even at 10 μg/mL, which suggests that Ara h 6 could have unique IgE-binding epitope(s). This is supportive of the report by Lehmann et al., who performed an enzyme allergosorbent test and found that maximal inhibition by

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recombinant Ara h 2 and recombinant Ara h 6 was only 70 and 60%, respectively.[15,45]

Ara h 2 consistently induced higher levels of histamine release than Ara h 6 in basophil studies. In general, a 10-fold lower concentration of Ara h 2 than Ara h 6 was required to induce the same amount of histamine release. Similarly, Ara h 2 was recently found to be more potent than Ara h 6 using the RBL SX-38 cells assay, although the magnitude of the difference was less than we report here with the stripped basophil assay.[46] Ara h 2 also elicited a higher magnitude of mediator release at lower concentrations than Ara h 6.[15] However, as the allergen concentration increased, Ara h 6 induced the release of higher percentages of β-hexosaminidase than Ara h 2.[47]

While most published data suggest that Ara h 2 is more potent than Ara h 6, there are exceptions. Ara h 6 had a higher seroprevalence (83.3%) than Ara h 2 (72.2%) in our study that involved testing 54 sera from peanut-allergic patients. In another study, Ara h 6 was shown to produce a stronger Th2 response than Ara h 2 in peripheral blood mononuclear cells of peanut-allergic children.[48] The depletion of Ara h 2 or Ara h 6 alone from crude peanut extract did not cause a significant reduction in the maximal net level of mediator release from SBX-38 cells, but the removal of both allergens decreased effector activity by approximately 20%.[49] Previously, Ara h 2 and Ara h 6 together were shown to be responsible for over 60% of the effector activity.[50] Furthermore, *in vivo* studies using murine models showed that desensitization with the Ara h 2/Ara h 6 mixture and crude peanut extract produced comparable results.[51,52]

Peanut allergy associated with sensitization to storage proteins (Ara h 1, Ara h 2, Ara h 3, Ara h 6, and Ara h 7) presents the most serious form of peanut allergy.[53] While Ara h 1 and Ara h 3 are the most abundant storage proteins in peanut (11−31 and 38−76% of protein content in peanut extracts, respectively), patients with peanut allergy recognize predominantly Ara h 2 and Ara h 6.[13,14,54] Molecular diagnostics have shown that the combined results of IgE reactivity to the two storage proteins Ara h 2 and Ara h 6 yielded the highest diagnostic sensitivity and specificity for detecting clinically evident peanut allergy.[13] By themselves, Ara h 2 and Ara h 6 had high diagnostic sensitivity and specificity compared to Ara h 1 and Ara h 3, but together, the two 2S albumin proteins were able to predict peanut allergy with 98% sensitivity and 85% specificity at a predictive threshold of 0.1 kU/L. The addition of specific IgE tests for other storage proteins, such as Ara h 6, or Ara h1, and Ara h 3, will increase specificity and sensitivity of molecular diagnosis, because not all patients with clinical peanut allergy show sensitization to Ara h 2.[32,53,55] Thus, purified nAra h 6 should be considered an important diagnostic reagent for both *in vitro* and *in vivo* assays that, together with nAra h 2 will improve diagnostic, immunologic, and biologic assays used in the investigation of peanut allergy, T-cell studies, and mouse models of asthma.

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Notes

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ABBREVIATIONS USED

nAra h 6, natural Ara h 6; nAra h 2, natural Ara h 2; T-Ara h 6, TNO-produced Ara h 6 used as a reference; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; LC−MS/MS, liquid chromatography−tandem mass spectrometry; ELISA, enzyme-linked immunosorbent assay; WHO, World Health Organization; PR-10, pathogenesis-related class 10 protein; PVDF, polyvinylidene difluoride; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)

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De-glycosylated recombinant Ara h 8 – an integral component for molecular diagnosis of peanut allergy

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RATIONALE

Natural Ara h 8, a Bet v 1-homologous peanut allergen involved in the cross-reaction with birch pollen has been associated with oral allergy syndrome (OAS) in birch pollen-allergic individuals (Ref. 1). The structure, which has recently been solved, shows an overall fold similar to Bet v 1 (Fig.1, Ref.3). Ara h 8 is a low abundance, unstable peanut protein that is difficult to purify from peanut (Ref. 2). Our goal was to produce recombinant Ara h 8 in *Pichia pastoris* for use in molecular diagnosis of peanut allergy.

METHODS

Recombinant Ara h 8.0101 and de-glycosylated rAra h 8 were expressed in *Pichia pastoris*. The glycosylation site was silenced by substituting asparagine at position 80 with glutamine (N80Q), thus disrupting the N-glycosylation motif in Ara h 8 cDNA. Recombinant Ara h 8 and N80Q-enriched ammonium sulfate fractions were purified by anion exchange chromatography followed by gel filtration chromatography. Identity of Ara h 8 was confirmed by liquid chromatography - tandem mass spectrometry. N80Q was compared to rAra h 8 and natural Bet v 1 by SDS-PAGE and IgE Ab ELISA.

Fig. 1

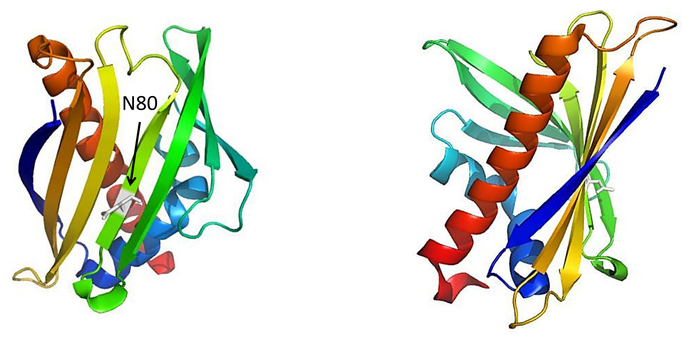

Fig. 1: Ribbon representation of Ara h 8. The N-glycosylation site (N80) is shown in grey.

Courtesy of Dr. Anna Pomés

RESULTS

Purification of recombinant Ara h 8: Recombinant Ara h 8 (rAra h 8) was expressed in *Pichia pastoris,* enriched using ammonium sulfate precipitation, and purified using anion exchange chromatography (Fig. 2A) and gel filtration chromatography (Fig.2B). Purified rAra h 8 presented as a mixture of hyper-glycosylated (19kD) and non-glycosylated forms (17kD) on silver-stained SDS-PAGE under non-reducing conditions (Fig.2C).

Fig. 2

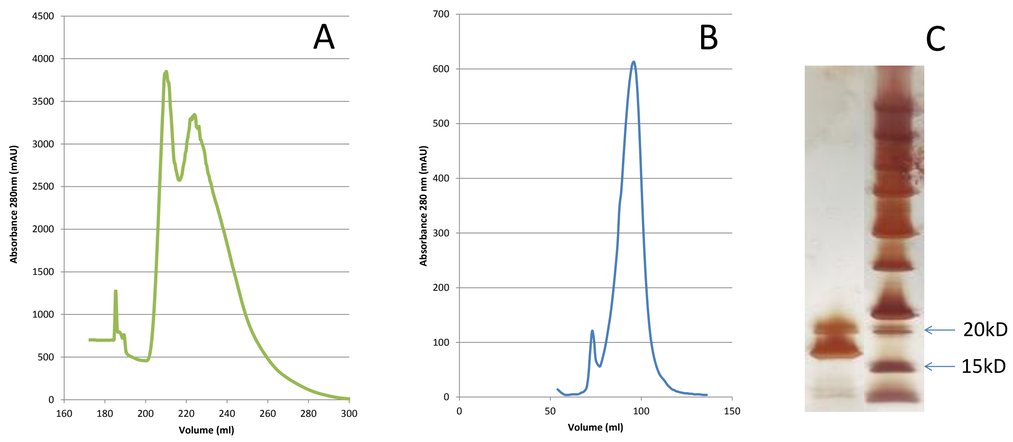

Fig. 2 Chromatography profiles and SDS-PAGE of rAra h 8. 2A: Elution profile of anion exchange chromatography of rAra h 8. Fractions of the anion exchange elution peak were further purified by gel-filtration chromatography. 2B: Elution profile of rAra h 8 gel filtration chromatography. Fractions of the elution peak were pooled and analyzed by SDS-PAGE. 2C: SDS-PAGE under non-reducing conditions followed by silver-staining. Lane 1:rAra h 8. Lane M: Marker.

Mass Spectrometry: Peptides identified after tryptic digest via LC/MS-MS confirmed the identity of Ara h 8 with a sequence coverage of ~80% in both the 19kD band and the 17 kD band (Fig.3). The lack of sequence coverage around the glycosylation motif N80 indicates the presence of glycans.

Fig. 3

tr|Q6VT83|Q6VT83_ARAHY (100%), 16,952.8 Da
Ara h 8 allergen OS=Arachis hypogaea PE=2 SV=1
23 exclusive unique peptides, 49 exclusive unique spectra, 180 total spectra, 124/157 amino acids (79% coverage)

M G V F T F E D E I T S T V P P A K L Y N A M K D A D S I T P K I I D D V K S V E I V E G N G G P G
T I K K L T I V E D G E T K F I L H K V E S I D E A N Y A Y N Y S V V G G V A L P P T A E K I T F E
T K L V E G P N G G S I G K L T L K Y H T K G D A K P D E E E L K K G K A K G E G L F R A I E G Y V
L A N P T Q Y

Fig. 3: LC/MS_MS peptides of rAra h 8 after tryptic digest.

Fig. 4

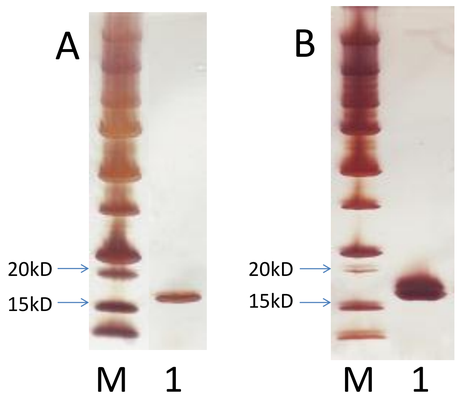

Fig. 4: SDS-PAGE of de-glycosylated rAra h 8 (A, Lane 1) and natural Bet v 1 (B, Lane 1) followed by silver staining. Lane M: Marker.

De-glycosylated rAra h 8: Removal of the glycosylation site (N80) results in expression of non-glycosylated Ara h 8 only (4A, Lane 1). The molecular weight of 17kD is similar to the size of natural Bet v 1 (4B, Lane 1).

IgE reactivity of rAra h 8: 22 of 67 (33%) sera from birch pollen-allergic individuals tested using a recombinant IgE ELISA showed IgE reactivity to rAra h 8 and 25 of 67 (37%) reacted with de-glycosylated rAra h 8 (N80Q). However, IgE reactivity to the glycosylated rAra h 8 was on average 70% lower than IgE reactivity to the de-glycosylated rAra h 8 (N80Q), except for two sera. This suggests that glycosylation at N80 interferes with IgE binding and that key IgE binding epitope(s) are near this amino acid residue. IgE reactivity to rAra h 8 was also tested using sera from peanut-allergic individuals. Four of 45 (7%) and five of 45 (9%) peanut sera tested, reacted with rAra h 8 and de-glycosylated rAra h 8, respectively, confirming that Ara h 8 represents a minor peanut allergen.

Fig. 5

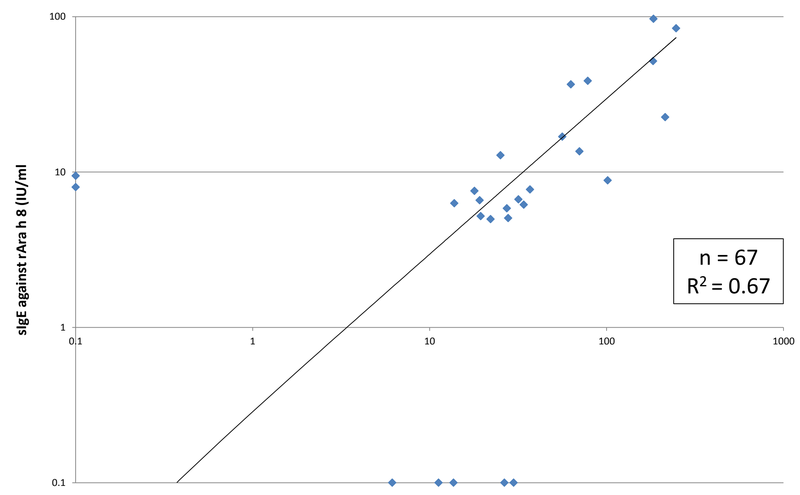

Fig 5. : Correlation between IgE Ab binding to rAra h 8 and rAra h 8 N80Q by recombinant IgE ELISA

CONCLUSIONS

Disruption of the N-glycosylation motif in rAra h 8 removed the hyper-glycosylated forms in *Pichia pastoris* expressed rAra h 8 and increased IgE antibody binding. These results indicate that key epitope(s) for IgE antibody binding are either adjacent to or overlap the glycosylation motif at residue N80. De-glycosylated rAra h 8 will distinguish peanut-sensitized individuals at risk of severe symptoms from those likely to have milder symptoms as a result of primary sensitization to homologous tree pollen. Thus, rAra h 8 represents a critical molecular diagnostic for risk assessment of severe reactions to peanut.

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* In relation to this presentation, I declare the following real or perceived conflict of interest: Employee of INDOOR Biotechnologies Inc. Research funded by INDOOR Biotechnologies Inc.

#803 A Sensitive Immunoassay for Invertebrate Tropomyosin Allergen in Foods, Inhalants, Ticks and Worms

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RATIONALE

Tropomyosin is a heat stable, major allergen of shellfish and other crustacea. Dust mite and cockroach tropomyosins are also allergenic. Cross-reactivity between tropomyosins may cause allergic symptoms related to allergen exposure.

Our aim was to develop a sensitive ELISA that could be used to measure tropomyosin for both exposure assessment and allergen standardization.

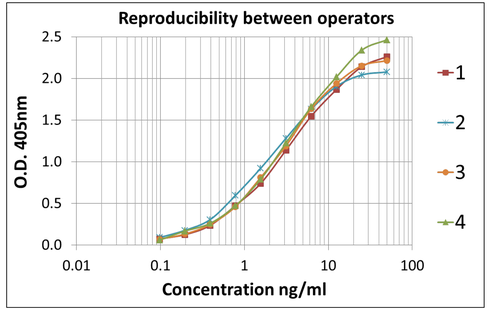
METHODS

A two-site ELISA was developed using a monoclonal antibody to Der p 10, which cross reacts with shellfish tropomyosin, and with a polyclonal anti-shrimp tropomyosin for detection. The assay was calibrated using purified natural shrimp tropomyosin, with protein content determined by amino acid analysis.

The tropomyosin assay was evaluated using commercially available shellfish (shrimp, crab, clam, lobster, oyster, scallop), dust mite (*Dermatophagoides pteronyssinus* and *farinae*) and cockroach extracts from major allergen manufacturers. In addition extracts were prepared from local insects, arachnids and home-cooked shellfish specimens.

RESULTS

- The assay was sensitive with a Lower Limit of Detection (LLOD) of 0.2ng/ml.
- The Tropomyosin Standard ranged from 0.1-50ng/ml.
- Results were reproducible between operators (n=4).



- The tropomyosin ELISA predominately detects invertebrate tropomyosins in shellfish, dust mites, tick, cockroach, meal worms, insects and arachnids.
- Extracts of fish, mammals, and birds did not react in the assay (<0.20ng/ml).

Shellfish	TM µg/ml
Shrimp (n=4)	21 – 297
Crab (3)	43 – 117
Clam	1.5
Lobster	37
Lobster (cooked)	1586 (µg/g)
Crawfish (cooked)	1449 (µg/g)
Oyster	0.24
Scallop	0.10
Mixed Shellfish (2)	1 – 25

Mites, Tick, Worms	TM µg/ml
D. pteronyssinus (4)	5 – 7
D. farinae (2)	4 – 5
B. tropicalis	12 (µg/g)
C. arcuatus	2 (µg/g)
Tick	3
Yellow Meal Worm (5)	13 – 38 (µg/g)

Insects/Arachnids	TM µg/g
American CR (3)	0.2 – 10 (µg/ml)
German CR (3)	4 – 9 (µg/ml)
Ladybug	217
Stink bug	47
Cicada	223
Moth	690
Spiders (3)	3 – 476
Cave cricket	0.40

CONCLUSIONS

- A sensitive ELISA (LLOD=0.2ng/ml) with defined specificity for invertebrate tropomyosins has been developed.
- This assay will allow standardization of tropomyosin levels in diagnostic or therapeutic allergen extracts.
- The assay may also be useful for providing a method for monitoring tropomyosin exposure in the environment and as a potential indicator of shellfish contamination in the food industry.

A Two-site Immunoassay for Quantification of Peanut Allergen Ara h 8

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RATIONALE

Approximately 0.5-2.0% of the world's population is sensitized to at least one of the seventeen peanut allergens currently registered in the WHO/IUIS database.[1] Peanut allergy is diagnosed, in part, by administering skin prick tests using peanut extracts. These extracts often comprise multiple allergens, and a large percentage of individuals diagnosed with peanut allergy may not be at risk for a systemic reaction due to sensitivity to a minor allergen(s) in the complex extract, or cross-reactivity with other allergens.[2]

The minor peanut allergen Ara h 8 is considered an important marker for sensitization. Although Ara h 8 typically does not present a significant health risk, it is a potential trigger for oral allergy syndrome (OAS) in birch pollen allergic individuals due to structural homology with Bet v 1.[3] Our aim was to develop a sensitive immunoassay for the detection of Ara h 8.

METHODS

Recombinant Ara h 8 (rAra h 8) produced by Indoor Biotechnologies was used to immunize mice and rabbits for development of monoclonal and polyclonal antibodies, respectively. Antibodies were screened by ELISA for reactivity to peanut allergens Ara h 1, 2, 3, 6, and 8, as well as to Bet v 1. A two-site ELISA was developed using a monoclonal antibody for capture and purified polyclonal IgG antibody for detection. An assay calibration standard was formulated using rAra h 8 with a measured concentration of 2,500ngmL (determined by Advanced Protein Assay, Cytoskeleton, Inc., Denver, CO, USA).

RESULTS

Monoclonal Antibody Specificity

4G6

Ara h 1	0.08	0.08	0.08	0.07	0.07	0.08	0.07	0.07	0.07	0.07	0.07	0.07
Ara h 2	0.08	0.07	0.08	0.07	0.07	0.08	0.07	0.07	0.07	0.07	0.07	0.07
Ara h 3	0.08	0.07	0.07	0.07	0.08	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Ara h 6	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Ara h 8	2.14	2.11	2.23	2.11	2.03	1.68	1.42	0.97	0.63	0.39	0.25	0.07
Bet v 1	1.50	1.40	1.38	1.27	1.20	1.17	0.80	0.56	0.42	0.29	0.20	0.07

4F7

Ara h 1	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Ara h 2	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Ara h 3	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Ara h 6	0.08	0.08	0.08	0.07	0.08	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Ara h 8	0.44	0.26	0.17	0.12	0.11	0.09	0.08	0.08	0.08	0.08	0.08	0.08
Bet v 1	0.28	0.18	0.12	0.10	0.09	0.09	0.08	0.08	0.08	0.08	0.08	0.08

Polyclonal Antibody Specificity

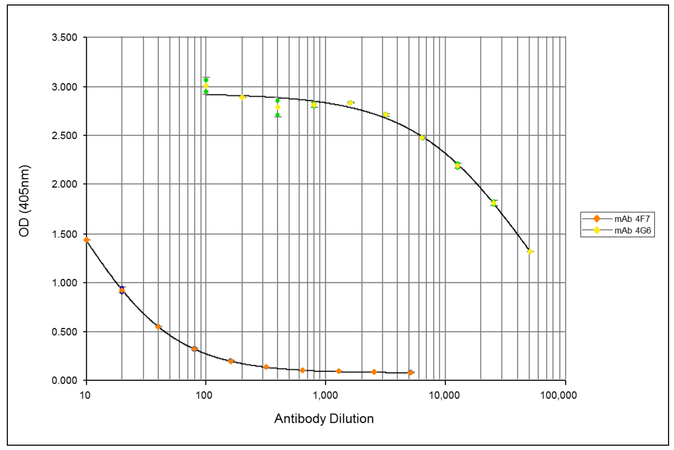
Ara h 1	0.15	0.10	0.09	0.08	0.07	0.07	0.07	0.07	0.06	0.07	0.12	0.07
Ara h 2	0.19	0.13	0.10	0.08	0.07	0.07	0.07	0.07	0.07	0.07	0.14	0.07
Ara h 3	0.55	0.29	0.19	0.13	0.10	0.08	0.08	0.07	0.07	0.07	0.45	0.07
Ara h 6	0.15	0.10	0.08	0.08	0.07	0.07	0.07	0.07	0.07	0.17	0.12	0.07
Ara h 8	2.68	2.36	1.98	1.53	1.06	0.65	0.40	0.25	0.16	0.12	0.13	0.07
Bet v 1	0.36	0.21	0.15	0.10	0.09	0.08	0.07	0.07	0.09	0.10	0.14	0.07

Results (OD 405nm) from ELISA screening of purified peanut allergens and birch pollen allergen, Bet v 1. Monoclonal antibodies 4G6 and 4F7 were specific for Ara h 8 amongst peanut allergens, and were cross-reactive with Bet v 1. Minor cross-reactivity of the polyclonal antibody was observed for all peanut allergens and Bet v 1.

Assay background values are shown in red type. Values in green type are results for pre-immunization sera for the polyclonal antibody.

RESULTS cont.

Ara h 8 ELISA Development



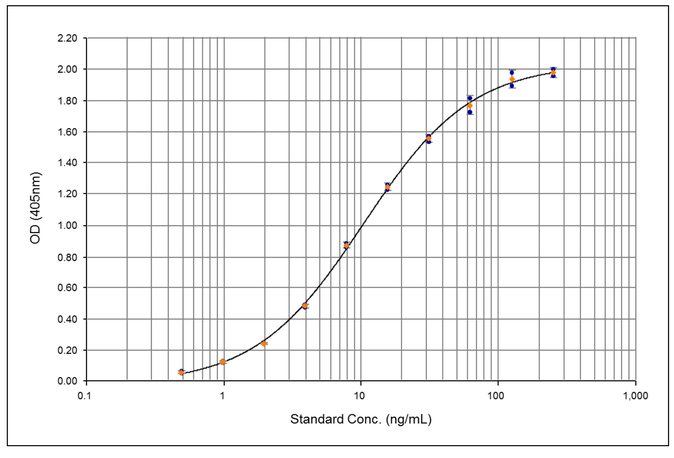
Monoclonal antibody reactivity with Ara h 8: mAb 4G6 reacted very strongly, but mAb 4F7 did not perform well enough for practical use in a two-site immunoassay.

Ara h 8 ELISA Specificity

Ara h 1	0.09	0.10	0.09	0.09	0.10	0.10	0.09	0.10	0.10	0.10	0.10	0.10
Ara h 2	0.09	0.09	0.10	0.10	0.10	0.10	0.10	0.09	0.10	0.10	0.10	0.10
Ara h 3	0.10	0.09	0.09	0.10	0.10	0.10	0.10	0.09	0.10	0.09	0.10	0.10
Ara h 6	0.09	0.09	0.10	0.10	0.10	0.09	0.10	0.09	0.10	0.09	0.10	0.10
Ara h 8	2.02	1.98	1.93	1.77	1.53	1.18	0.85	0.51	0.31	0.20	0.15	0.10
Bet v 1	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.11

Evaluation of peanut allergens and Bet v 1 in the two-site assay using mAb 4G6 for capture and pAb for detection demonstrates specificity for Ara h 8.

ELISA Calibration Curve



Optimized calibration curve for the two-site Ara h 8 ELISA using mAb 4G6 for capture and purified pAb for detection.

- cross-reactivity with Bet v 1 overcome at antibody concentrations used in the assay
- standard range: 250-0.49ng/mL
- limit of quantification: 1.95ng/mL

Ara h 8 Levels in Peanut Foods

Butters	Description	ug/g
Peanut #1	NIST SRM 2387	1.0
Peanut #2	Reduced fat/calorie	<0.2
Peanut #3	Organic raw peanut	31.5
Peanut #4	Organic crunchy	1.1
Peanut #5	Creamy	3.2
Peanut #6	Fresh ground	2.2
Peanut #7	Natural chunky	0.5
Peanut #8	Creamy whipped	1.9
Peanut #9	Natural crunchy	1.4
Peanut #10	Natural creamy	0.7
Hazelnut	Chocolate hazlenut blend	<0.2
Soy	Roasted soybean butter	<0.2
Almond	Dry roasted	<0.2
Cashew	Organic raw cashew	<0.2

Flours/powders	Description	ug/g
Peanut #1	Dry roasted	41.9
Peanut #2	12% fat, light roast	0.8
Peanut #3	28% fat, light roast	10.7
Bamba	Peanut puff	0.6
Chickpea	Whole flour	<0.2
Lupini	Bean powder	<0.2
Oils	**Description**	**ug/mL**
Peanut #1	Refined, organic	<0.02
Peanut #2	Roasted, unfiltered	<0.02
Extracts	**Description**	**ug/mL**
Peanut #1	Greer	5.1
Peanut #2	ALK	1.5
Birch pollen	Greer	<0.02
Purified Bet v 1	Indoor Biotechnologies	<0.02

The concentration of Ara h 8 in peanut products varies significantly, even amongst similar types of samples. This was also observed when the same set of samples was tested for peanut allergens Ara h 1, 2, and 6 (results not shown). Non-peanut products were not detectable in the Ara h 8 ELISA, demonstrating specificity for Ara h 8.

A panel of house dust extracts with detectable levels of Ara h 6 was also tested using the Ara h 8 ELISA. Results for all samples were below the detection limit of the assay.

CONCLUSIONS

- A specific, sensitive two-site ELISA for the quantification of peanut allergen Ara h 8 has been developed.
- Applications include:
  - measurement of Ara h 8 in food products and peanut extracts
  - standardization of allergy diagnostic and therapeutic products
- The assay may help distinguish between sensitivity to Ara h 8 and Bet v 1, leading to a better understanding of allergen cross-reactivity and oral allergy syndrome.

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Doses of Major Allergens in Peanut Associated with Oral Tolerance

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RATIONALE

The LEAP study showed that peanut consumption in early life dramatically reduced the prevalence of peanut allergy among high risk children. The preferred peanut snack used for the study was Bamba, a corn puff containing ~50% peanut. Our objectives were to compare Ara h 1, Ara h 2 and Ara h 6 levels in Bamba and to estimate the weekly doses of specific peanut allergens associated with oral tolerance.

METHODS

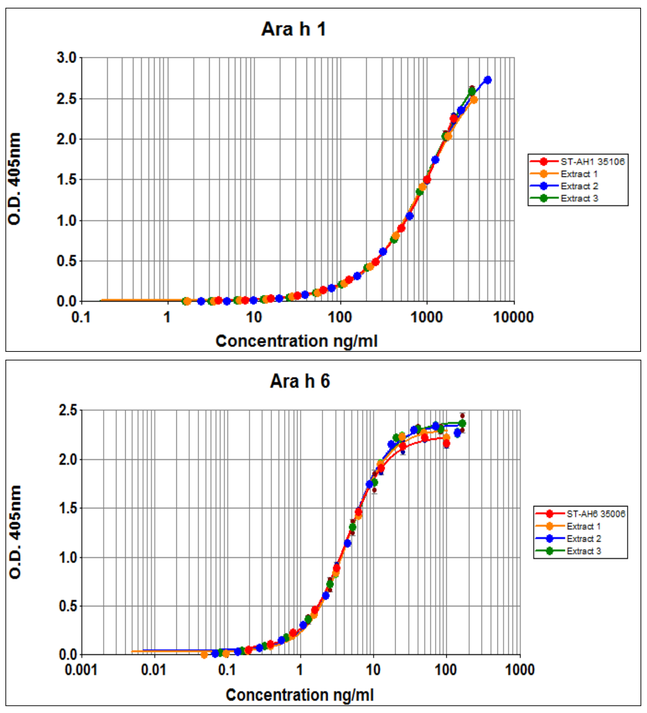
Extracts of Bamba (100 mg/sample) from either the UK (n=8) or US (n=8) and various types of the German peanut snack (n=10), ErdnußLocken, or 25 individual Bamba sticks, were analyzed by ELISA for Ara h 1, Ara h 2 and Ara h 6 using purified natural allergen standards. The limits of detection of the ELISA were: Ara h 1, 32ng/ml; Ara h 2, 2ng/ml and Ara h 6, 0.8ng/ml (Figure 1). Allergen consumption in the LEAP study was estimated by calculating the amount of allergen/Bamba stick and extrapolating from the median peanut consumption reported in the study (7.7g peanut protein/week).

RESULTS

- Peanut butter contained more Ara h 1 than other peanut allergens and typically had 2-4 fold more Ara h 1 than Ara h 2 or Ara h 6 while peanut flour showed up to 20 fold higher levels of Ara h 2 and Ara h 6 compared to Ara h 1 (Table 1,2).
- The absolute amounts of peanut allergens in Bamba were remarkably consistent between the US and UK: n=16, Ara h 1, 2,388µg/g (12%CV); Ara h 2, 1,988µg/g (15%); and Ara h 6, 2,341µg/g (16%); ~1:1:1 ratio (Figure 2).
- The German ErdnußLocken had 3-4 fold lower amounts of peanut allergen compared to Bamba (Figure 2).
- Individual Bamba sticks contained similar levels of peanut allergens: n=25, Ara h 1, 1,499µg/g (14%CV); Ara h 2, 2,169µg/g (12%CV); and Ara h 6, 2,295µg/g (14%CV).
- Peanut butters and flours used to prepare oral immunuotherapy (OIT) doses used in clinical trials at UNC and UVA had 2-10 fold more Ara h 2 than Ara h 1 (Table 3).
- Median weekly doses of allergens were calculated based on consumption of 80 Bamba sticks (equivalent to 7.7g peanut protein) and amounted to: 83mg Ara h 1, 120mg Ara h 2 and 127mg Ara h 6 (total 330mg/week) (Table 4).

RESULTS

Figure 1. ELISA Control Curves



Limits of Detection (LOD)		
Ara h 1	Ara h 2	Ara h 6
32ng/ml	2ng/ml	0.8ng/ml

Figure 2. Specific Peanut Allergen Levels in Bamba

Bamba	Ara h 1 µg/g (CV)	Ara h 2 µg/g (CV)	Ara h 6 µg/g (CV)
Total (n=26)	1,757 (53%)	1,519 (45%)	1,699 (54%)
U.S. (n=8)	2,195 (11%)	1,953 (13%)	2,288 (13%)
U.K. (n=8)	2,582 (8%)	2,023 (18%)	2,393 (19%)
Germany (n=10)	626 (77%)	687 (29%)	557 (18%)

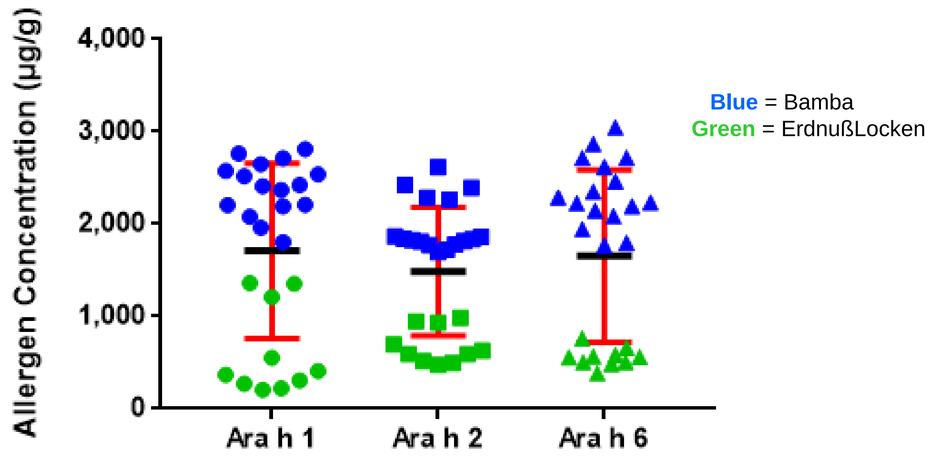


Table 1. Allergen Ratios: Peanut Butter, Flour and Bamba

	Ara h 1:Ara h 2*	Ara h 1:Ara h 6*
Peanut Butter (n=16)	2.0 ± 0.6	2.7 ± 0.7
Peanut Flour (n=11)	0.5 ± 0.4	0.7 ± 0.6
Bamba (n=15)	1.3 ± 0.1	1.0 ± 0.1

Table 2. Allergen Levels in Peanut Butter and Flour

Butter	Description	Ara h 1 (µg/g)	Ara h 2 (µg/g)	Ara h 6 (µg/g)	Total Peanut (ppm)
NIST SRM 2387	Reference Standard	11,272	2,522	2,036	5,571,182
Smucker's	Natural	10,321	4,131	3,480	4,230,551
Whole Foods	Crunchy	21,406	7,859	5,786	4,705,309
Walmart	Natural Crunchy	10,323	4,568	3,439	2,018,995
Flour	**Description**	**Ara h 1 (µg/g)**	**Ara h 2 (µg/g)**	**Ara h 6 (µg/g)**	**Total Peanut (ppm)**
PP	12% Fat Light Roast	13,473	7,504	4,591	6,093,056
OVBM	12% Fat Light Roast	3,096	7,091	5,782	1,675,889
OVBM	12% Fat Dark Roast	122	1,816	1,258	>1,000
Golden	12% Fat Light Roast	6,280	8,161	6,385	3,626,987
Golden	28% Fat Dark Roast	34	787	624	2,762,537
Nuts.com		5,262	14,631	14,564	>10,000,000

Table 3. Allergen Levels in Oral Immunotherapy Preparations

	Sample	Ara h 1 (µg/ml)	Ara h 2 (µg/ml)	Ara h 6 (µg/ml)	Ara h 2:1 (µg/ml)
UNC*	1	394	1,187	1,104	3.0
	2	505	1,431	1,448	2.8
	3	358	1,879	1,891	5.2
UVA**	-20°C Stock	487	5,270	8,093	10.8
	-20°C Solution	43	87	95	2.0
	4°C Solution	<0.16	<0.01	87	

**The -20°C and 4°C solutions were diluted to 1mg/ml based on the total protein measurement of the stock preparation.
Kindly provided by Drs. Brian Vickery, UNC and Dr. Scott Commins, UVA**.*

Table 4. Estimated Doses of Peanut Allergens in Bamba Administered During the LEAP Study

Dose		Ara h 1* (mg)	Ara h 2* (mg)	Ara h 6* (mg)
Mean allergen/stick		1.03	1.48	1.57
21 sticks**	One serving	21.6	31.1	33
80 sticks***	Median weekly dose	83.2	119.7	127
80 sticks x 52 weeks***	12 months consumption	4,326	6,224	6,604

*Mean of 25 individual sticks analysed for each allergen; mean stick weight = 0.68g
**One serving as described in WAO Consensus Communication; 21 sticks = ~17g Bamba = 2g peanut protein
***Median weekly dose, equivalent to 7.7g peanut protein

References:
1.Du Toit et al, Randomized trial of peanut consumption in infants at risk for peanut allergy. New Eng J Med 2015; 372:803-13.
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CONCLUSIONS

- Unlike other peanut food products, Bamba is a reproducible and consistent formulation of peanut allergens.
- The three major allergens are present in uniform amounts in Bamba, but vary greatly in level and composition in peanut foods.
- For the first time, the results provide target doses of specific peanut allergens that are associated with prevention of peanut allergy and which, by extension, could apply to the induction of tolerance to other food allergens.
- Specific allergen content should be taken into account when using food formulations or specific foods for clinical trials of oral immunotherapy or prophylactic interventions.

Development and Application of Quantitative Immunoassays for Major Milk Allergens Bos d 5 (β-lactoglobulin) and Bos d 11 (β-casein)

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INTRODUCTION

In order to help allergic patients manage often severe symptoms, food manufacturers are required to list allergens on their products and researchers are working to develop effective immunotherapies. Due to limitations of the existing tools, precise quantification and standardisation of milk allergens in food, therapeutic and diagnostic products can be difficult.

Aim: We sought to develop accurate, sensitive and reliable assays that would enable quantification of multiple milk allergens.

MATERIALS AND METHODS

- Allergen specific mAbs were developed against Native Bos d 5, Denatured Bos d 5 and Bos d 11 (Table 1).
- These mAbs were used to develop allergen specific ELISA and Multiplex (Figure 1) immunoassays.
- Purified natural allergens (Figure 2) were used to generate a standard curve for each protein.
- Detection of the target allergens was accomplished using biotinylated specific mAbs antibodies (Table 1). and streptavidin conjugated fluorochrome.
- Allergen content was measured using multiplex, in various sample types including iFAAM reference samples, milk powder, chocolate dessert, cookie and chocolate bar. The results were compared to ELISA.

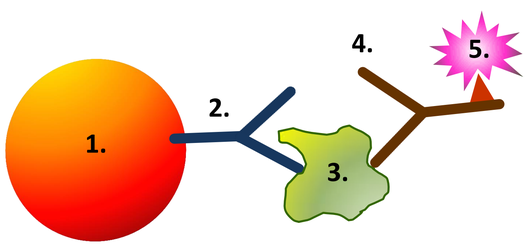

Figure 1. 1. Fluorescent microsphere; 2. Allergen specific antibody coupled to the bead; 3. Target protein; 4. Allergen specific biotinylated detection antibody; 5. Streptavidin-PE.

Target food	Target protein	Standard	Capture Antibody	Detection Antibody
Milk	Native Bos d 5	N-Bos d 5	97N	117N
	Denatured Bos d 5*	D-Bos d 5	74R	92R
	Bos d 11	Bos d 11	CC11	VB1C

Table 1. Milk proteins and antibody pairs used for development of the milk immunoassays. *nBos d 5 was denatured via alkylation to create dBos d 5

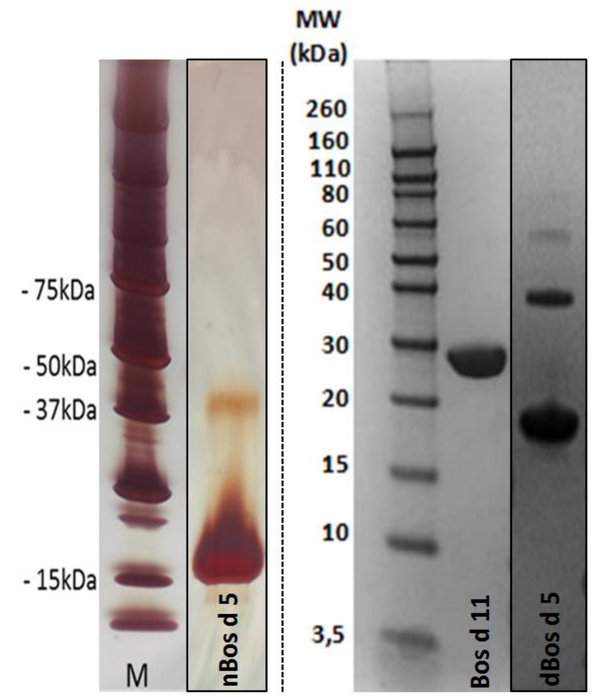

Figure 2. SDS-PAGE diagrams of purified native Bos d 5, denatured Bos d 5, and Bos d 11 (β-casein) used as standards in milk immunoassays.

RESULTS

- The food multiplex assay was able to measure multiple allergens in a small (<50µl), single sample.
- Lower limit of detection (LLOD) out of the assays was as low as 0.2 ng/ml for Native Bos d 5 (Table 2).
- Sensitivity of the multiplex assay was increased by up to 39-fold compared to ELISA.
- The multiplex food array produced reproducible results showing intra-assay CVs<8% and inter-assay CVs<15%.
- Multiplex standard curves range between 200-0.1 ng/ml for Native Bos d 5, 1000-0.49 ng/ml for Denatured Bos d 5 and 5000-2.44 ng/ml for Bos d 11 (Figure 3).
- There was a significant correlation between multiplex assays and ELISA for nBos d 5, dBos d 5 and Bos d 11 (Figure 4).

	ELISA LLOD (ng/ml)	Multiplex LLOD (ng/ml)	LLOD fold change	Multiplex intra-assay CV%*	Multiplex inter -assay CV%**	Multiplex assay parallelism***
Native Bos d 5	7.8	0.2	39.0	7	9	16
Denatured Bos d 5	7.8	2.0	3.9	8	5	24
Bos d 11	31.3	9.8	3.2	7	15	11

Table 2. Performance characteristics of the milk multiplex assay (*Mean CV% of average allergen concentration for duplicate samples run on the same plate; ** Mean CV% of average allergen concentration for samples analysed on at least two separate days; *** Mean CV% of average allergen concentration for at least 3 serial dilutions).

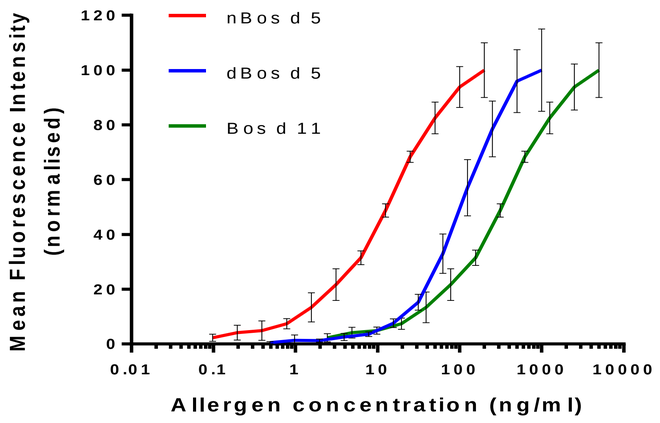

Figure 3. Standard curves for nBos d 5, dBos d 5 and Bos d 11 in the milk multiplex assays.

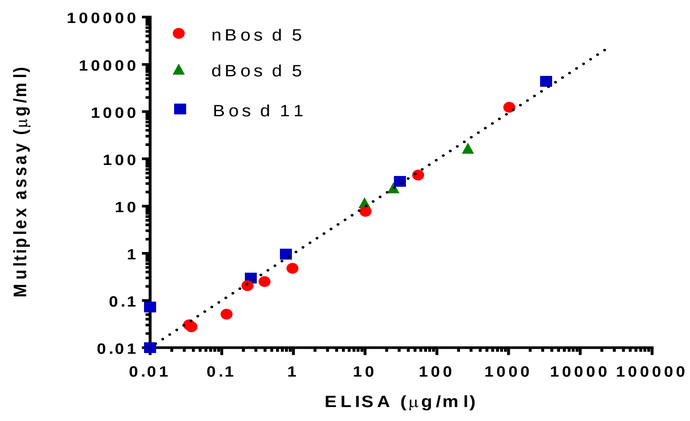

Figure 4. Correlation between results obtained using ELISA and multiplex milk assays. Analyzed samples were: allergen spikes, milk powder, research chocolate dessert with allergens and placebo; research chocolate bar and placebo; and research cookie.

CONCLUSIONS

- 'Proof-of-concept' ELISA and multiplex immunoassays for quantification of major milk allergens have been developed.
- The 'open-architecture' multiplex platform allows for addition of further allergens to the list of analytes and creation of a wider 'food-panel'.
- The array can provide a robust, rapid and cost effective alternative to existing methods for research, pharmaceutical, biotechnology and food industries.

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Disclosure: In relation to this poster I declare the following, real or perceived conflicts of interest: Ross Yarham, Anna Kuklinska-Pijanka, David Gillick, Elizabeth Young, Martin Chapman and James Hindley are employees of Indoor Biotechnologies. Karine Adel-Patient and Hervé Bernard are employees of INRA.