

## Lipid transfer protein (Ara h 9) as a new peanut allergen relevant for a Mediterranean allergic population

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**Background:** Nonspecific lipid transfer proteins (LTPs) represent potent pollen and food allergens. However, the allergenic properties of peanut LTP have not been studied. **Objective:** To identify LTP in peanut extract using sera from subjects with peanut allergy and Pru p 3-sensitized subjects from Southern Europe, clone and express this protein, and obtain information on the importance as allergen for these selected patients.

**Methods:** Peanut LTP (Ara h 9) was cloned and sequenced by using a combination of bioinformatic and molecular biology tools (PCR, immunoblotting, Basic Local Alignment Search Tool [BLAST] searches). The immunologic properties of Ara h 9, Ara h 1, Ara h 2, and Ara h 3 were studied by using sera from subjects with peanut and peach allergy from Italy by immunoblotting and allergen microarray technology.

**Results:** Two Ara h 9 isoforms—Ara h 9.01 and Ara h 9.02—were cloned and expressed. Ara h 9 represented a minor allergen for subjects with peanut allergy. However, including Ara h 9 as single component for serologic detection of sensitization to peanut by component-resolved diagnosis seems crucial, because the frequency of sensitization to the classic major peanut allergens Ara h 1, Ara h 2, and Ara h 3 was low in these patients from Southern Europe.

**Conclusion:** Ara h 9 is a new member of the LTP allergen family that seems to play an important role in peanut allergy for patients from the Mediterranean area. (*J Allergy Clin Immunol* 2009;124:771-8.)

**Key words:** Ara h 9, peanut allergy, lipid transfer protein, isoform, allergen

### Abbreviations used

IUIS: International Union of Immunological Societies

ISAC: Immuno solid-phase allergen chip

LTP: Lipid transfer protein

nAra h 9: Natural Ara h 9

rAra h 9: Recombinant Ara h 9

SPHIAa: Single point highest inhibition achievable assay

Today, 11 peanut allergens have been identified that are listed by the Allergen Nomenclature Sub-committee (<http://www.allergen.org>) of the International Union of Immunological Societies (IUIS). The first 8 peanut allergens (Ara h 1, Ara h 2, Ara h 3, Ara h 4, Ara h 5, Ara h 6, Ara h 7, Ara h 8) are characterized by molecular weights between 12 kd and 70 kd and isoelectric points between 4.6 and 7.2. Thus, alkaline extraction with ammonium hydrogen carbonate, pH 8.0,<sup>1</sup> or TRIS-buffered saline, pH 8.2,<sup>2</sup> is the first choice to prepare peanut extracts with representative amounts of these 8 peanut allergens. Even though lipid transfer proteins (LTPs) were identified in neutral and alkaline extracts with isoelectric points between 9.0 and 10.0, they might exist in low quantities, particularly if LTP content is already low in the food. Thus, the yield of LTP from peanut flour should increase under acidic extraction conditions. Nonspecific LTPs represent a class of proteins that contains potent pollen and food allergens.<sup>3-5</sup> Sequence identities among allergenic LTPs tend to be low (with some notable exceptions such as Pru p 3 and Mal d 3) even though their 3-dimensional structures defined by characteristic disulfide bonds are very similar. This makes it difficult to use existing information on amino acid and cDNA sequences of LTPs to clone and sequence still unknown allergenic LTPs.

The LTPs are proteins of 9 to 10 kd belonging to a multigene family. LTP-sensitized patients often show systemic reactions in addition to oral allergy syndrome, which is the predominant symptom in patients with birch pollen-related food allergies. The LTPs are very stable with regard to protease and thermal treatment, which is presumably a result of 4  $\alpha$ -helices stabilized by 4 disulfide bonds. It has been suggested that this stability is the cause of the more severe and systemic reactions, in contrast to other food allergens such as Bet v 1 homologs that are quickly digested in gastrointestinal fluid or pepsin.<sup>6-11</sup>

Allergic subjects from the Mediterranean populations often experience severe symptoms after consumption of fruits and vegetables. In this context, LTPs have been identified as allergens

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in peach (Pru p 3),<sup>12,13</sup> cherry (Pru av 3),<sup>8,14</sup> apple (Mal d 3),<sup>15,16</sup> and hazelnut (Cor a 8).<sup>17</sup> However, the existence of LTP in peanut extract was only indirectly described by Asero et al.<sup>18</sup> The question arises whether LTP can be identified in peanut by optimized extraction conditions based on the physico-chemical properties of this protein class.

Thus, the aim of this study was to identify LTP in peanut extract, and to clone and express it as recombinant protein using sera of a selected group of subjects with peanut allergy (Pru p 3-specific IgE) from Italy, a Mediterranean country where LTPs are important allergens. These studies were performed to set a first stage for the evaluation of the importance of peanut LTP in the relation to the classic peanut major allergens Ara h 1, Ara h 2, and Ara h 3 and minor allergens such as Ara h 8 (a Bet v 1-homolog protein) and profilins.

## METHODS

### Patients' sera

Sera included in this study (Table I) were collected at the Center for Clinical and Experimental Allergology, IDI-IRCCS (Rome, Italy). Three groups of patients were recruited (I, peanut allergy and Pru p 3-specific IgE antibodies; II, peanut allergy and no Pru p 3-specific IgE antibodies; III: no peanut allergy and Pru p 3-specific IgE antibodies) and grouped according to the allergic status in regard to peanut allergy and/or peach allergy (A, allergic to both peanut and peach; B, allergic only to peanut; C, allergic only to peach; D, no peanut and peach allergy). None of the patients underwent food challenge; only subjects reporting convincing relationships between ingestion of peanut or peach and onset of symptoms were included. Sera from subjects with Pru p 3-specific IgE and negative history of peanut allergy served as controls and were included in the study if they had eaten and tolerated peanuts within 6 months of the blood drawing. The use of patient serum samples tested in the study has been approved by the Institutional Ethic Committee, and patients signed an informed consent.

### Characteristics of patients and determination of specific IgE antibodies by CAP-FEIA and immuno solid-phase allergen chip

In Table I, general characteristics (sex, age) and individual symptoms reported after exposure to peanut and peach are summarized. Skin prick testing with peanut and peach extracts has been performed by a standard method.<sup>19</sup> Furthermore, Table I lists specific IgE antibodies to peanut and peach extracts measured by CAP-FEIA (Phadia, Uppsala, Sweden), IgE to natural purified peach LTP (nPru p 3), nAra h 1, nAra h 2, nAra h 3, Bet v 1 homologs (Ara h 8, Cor a 1.0401, Mal d 1, Pru p 1), and profilins (Bet v 2, Hev b 8, Mer a 1, Ole e 2, Phl p 12) measured by immuno solid-phase allergen chip (ISAC) technology (VBC-Genomics, Vienna, Austria). The IgE reactivities to the Bet v 1 homologs and the profilins were determined to study their role as sensitizing allergens. CAP-FEIA and ISAC were performed according to the manufacturer's instructions.

Values were expressed as kU/L; however, the results of all IgE measurements performed by ISAC were categorized as positive or negative (Table I). ISAC testing did not show any unspecific binding. Thus, ISAC testing has virtually no cutoff because control samples with very high IgE levels (>5000 IU/L) did not show any unspecific binding. Furthermore, each allergen was spotted 3 times on the chip. Positive results in triplicate ensure the correct identification of even weak IgE reactivities. Thus, any positive fluorescence in triplicate was scored as positive.

### ISAC microarray inhibition assay

Ten patients' sera with Pru p 3-specific and/or Cor a 8-specific (6/10) IgE antibodies were used in the inhibition assay. Individual sera were preincubated overnight with equal volumes of purified rAra h 9.0201 and nPru p 3 to final

concentrations of 650 µg/mL and 500 µg/mL, respectively. Buffer was used instead of allergen to obtain the total specific binding value. The remaining IgE reactivities to rCor a 8.0101 and nPru p 3 were measured on the ISAC 103 microarray. See also the Methods section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). The assay was run as a single point highest inhibition achievable assay (SPHIAa), and the percentage of inhibition was calculated. Specificity of the inhibition was assessed by incubation with only the detection antibody. Furthermore, SPHIAa-tested serum samples contained specific IgE to other allergens that are not cross-reactive with peanut allergens and peach and hazelnut LTP, so IgE reactivities to these allergens served as controls because they were not inhibited by Pru p 3 or Ara h 9.

### SDS-PAGE and immunoblot analysis

SDS-PAGE was performed according to Laemmli<sup>20</sup> (15% T [total concentration of acrylamide and bis-acrylamide in percent] and 2.67% C [ratio of cross-linker (bis-acrylamide) and total acrylamide in percent]). For immunoblot analysis, separated proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Schwalbach, Germany) by semidry blotting for 30 minutes at 0.8 mA/cm<sup>2</sup>.<sup>21</sup> The membranes were blocked for 2 hours in TRIS-buffered saline containing Tween 20 (100 mmol/L TRIS/HCl, 100 mmol/L NaCl, 2.5 mmol/L MgCl<sub>2</sub>, 0.05% Tween 20, pH 7.4) containing 5% nonfat dry milk. The membranes were incubated overnight with His-tag-specific mAb (Penta-His antibody, 1:5000; Qiagen, Hilden, Germany), or patients' sera (1:20), diluted in TRIS-buffered saline containing Tween 20. Bound antibodies were detected with alkaline phosphatase-labeled (AP)-conjugated secondary antibodies (anti-mouse IgG + IgM, 1:10,000; Dianova, Hamburg, Germany; or anti-human IgE, 1:2000; Allergopharma, Reinbek, Germany) for 2 hours. Cor a 8 or peanut LTP was detected by rabbit anti-Cor a 8 antiserum (1:40,000) via AP-conjugated goat anti-rabbit serum (1:10,000, Dianova). Bound antibodies were visualized by using the nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate NBT/BCIP chromogen/substrate system.<sup>22</sup> The intensities of the reactivities to Ara h 9 were categorized as positive (+) and negative (−).

### N-terminal sequencing

Proteins separated by SDS-PAGE were transferred by semidry blotting onto polyvinylidene difluoride membrane, and Coomassie-stained.<sup>23</sup> IgE-reactive bands were cut from the membrane, and microsequencing was performed according to Edman<sup>23</sup> on a Procise protein sequencer (Applied Biosystems, Darmstadt, Germany).<sup>24</sup>

### Cloning, expression, and purification of recombinant Ara h 9

Total RNA was isolated from flour of raw, unroasted peanuts (Spanish Type; Max Kiene, Hamburg, Germany) using the SV Total RNA Isolation System (Promega, Mannheim, Germany). To generate cDNA, the First Strand cDNA Synthesis Kit (GE Healthcare, Munich, Germany) was used in combination with the Not I d(T)<sub>18</sub>-Primer (AACTGGAAGAATTCGC GGCCGCAGGAA(T)<sub>18</sub>; GE Healthcare). Because cDNA sequences of peanut LTPs were unknown, cDNAs of LTPs from cherry (AF221501), peach (AY792996), strawberry (DQ066732), hazelnut (AF329829), and lentil (AY793556) were aligned, and a sequence stretch with high sequence identity in the 3' region was found (see this article's Fig E1 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). A forward primer (LTP\_hom\_for: CATTCCTTACAAGATCAGCACCTC) and the Not I d(T)<sub>18</sub> were used to amplify the 3' end of peanut LTP. Because the 5' rapid amplification of cDNA ends approach failed and did not give information on the 5' cDNA sequences, a nucleotide BLAST with the amplified 3' end against the expressed sequence tags database (<http://blast.ncbi.nlm.nih.gov>) restricted to sequences from *Arachis hypogaea* (taxid: 3818) was performed.

Several of the 189 full-length sequences of peanut LTP with sequence identities of 100% to the obtained 3' region were used to design a primer (LTP\_5'UTR\_for: CTTTCAACTCCAAAACACACTTAGC) in the 5' untranslated region. In combination with Not I d(T)<sub>18</sub> sequences, 2 peanut

LTP isoforms were amplified and cloned. The peanut LTPs were expressed as mature proteins (without signal peptide) in *Escherichia coli*.<sup>17,25</sup> The cDNA of a peanut LTP later designated Ara h 9.0101 was ligated in the *EcoR* I and *EcoR* V restriction sites of the expression vector pET32b and expressed as His-tagged thioredoxin fusion protein in *E coli* BL21(DE3) cells (Stratagene, La Jolla, Calif). For the second isoform, later designated Ara h 9.0201, a synthetic gene optimized for the expression in *E coli* (Geneart, Regensburg, Germany) was ligated in the *Nde* I and *Xho* I restriction sites of the expression vector pET23b and expressed as a C-terminally His-tagged protein in BL21(DE3) cells (Stratagene). After induction of expression with isopropyl-beta-D-thiogalactospyranoside (IPTG), the cells were pelleted by centrifugation and lysed under denaturing conditions. The recombinant proteins were purified by immobilized metal affinity chromatography using Talon Metal Affinity resin (Clontech, Mountain View, Calif) and the purity determined by SDS-PAGE. Finally, the proteins were dialyzed against distilled water, lyophilized, and stored at -20°C.

### Preparation of Ara h 9-enriched peanut extract

The Ara h 9-enriched peanut extract was prepared according to the method of Boldt et al<sup>26</sup> with one major modification. Because extract preparation with buffers with neutral and alkaline pH enriches acidic proteins, an acidic buffer (100 mmol/L ammonium acetate, pH 5.0) was selected for extraction, because LTPs have high isoelectric points. Furthermore, the acidic extract was fractionated by size exclusion chromatography on Superdex 75 (GE Healthcare, Freiburg, Germany). The Ara h 9-enriched fractions containing proteins with molecular weights below 20 kd were collected, dialyzed against distilled water overnight, lyophilized, and stored at -20°C.

## RESULTS

### Identification of allergenic LTP in peanut extract

Using the serum panel composed of sera from subjects with peanut allergy, peach allergy, and no allergy (total, n = 66; group I, peanut-allergic and Pru p 3-specific IgE, n = 25; group II, peanut-allergic and no Pru p 3-specific IgE, n = 17; group III, peanut nonallergic and Pru p 3-specific IgE, n = 24), no IgE-reactive bands with molecular weights below 10 kd were detectable in a peanut extract prepared under basic conditions (pH 8.0; see this article's Figs E2, A and E3, A, in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). IgE-reactive bands below 10 kd were found, however, when the peanut extract prepared under acidic conditions was used in the Western blot analysis (Fig E3, B). To enrich proteins with molecular weights below 20 kd from the acidic peanut extract, size exclusion chromatography was performed. Western blot analysis of this fraction demonstrated IgE-reactive bands in the range of 9 kd (Fig 1; Fig E3, C). The 14 N-terminal amino acids of this IgE-reactive 9 kd protein (K/G-S-X-G-Q-V-N-S-A-L-A-P-X-I; see this article's Fig E4 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)) were identified as belonging to LTPs; the unrestricted protein BLAST search returned only LTPs from various plant species for the first 100 sequences producing significant alignments.

Furthermore, with the exception of the first amino acid, the deduced amino acid sequences of recombinant peanut LTPs (Fig E4) were in agreement with the N-terminal amino acid sequence of natural peanut LTP.

### Peanut LTP (Ara h 9) represents a new allergen for subjects with peanut allergy

Nineteen (45.2%) sera of subjects with peanut allergy (n = 42) had IgE antibodies to Ara h 9 (Fig 1; Table II), characterizing Ara

h 9 as a minor allergen according to the rules of the IUIS Allergen Nomenclature Sub-committee. Of the 8 sera that were CAP-negative to peanut extract (Table I), none contained detectable amounts of Ara h 9-specific IgE antibodies, indicating that testing for Ara h 9-specific IgE antibodies may not increase the frequency of detection of peanut-specific sensitization. In sera of the 15 patients who had peach allergy but no peanut allergy (clinical status C), 8 tested positive to peanut extract by CAP-FEIA, and 2 tested positive to Ara h 9.

### Sensitizations to Ara h 9 and Pru p 3 are not disease-associated markers for allergies to peanut and peach

To correlate the association between IgE reactivities to peanut and peach, Table II was compiled from the data of Table I. Sensitizations to peach and peanut LTP was not strongly associated with the particular food allergy. Of the 26 subjects with peanut allergy but no peach allergy (group B), 38.5% reacted with Ara h 9, 46.2% with Pru p 3, and 30.8% with both of these LTPs. In the group of subjects who exclusively reported symptoms to peach (group C; n = 15) the frequency of sensitization to Ara h 9 was 26.7%, to Pru p 3 was 100%, and to both LTPs was 26.7%. Of the 16 subjects with both peanut and peach allergy (group A), 56.3% reacted with Ara h 9, 81.3% with Pru p 3, and 50.0% with both of these LTPs. IgE reactivities of all subjects with peanut allergy (groups A + B) and all subjects with peach allergy (groups A + C) to Ara h 9 (45.2% and 45.2%) and Ara h 9 plus Pru p 3 (38.1% and 38.7%) were identical, whereas the frequency of IgE reactivities to Pru p 3 alone was higher in the group of subjects with peach allergy (87.1%) than in the group of subjects with peanut allergy (59.5%).

In the studied population, the classic major peanut allergens Ara h 1, Ara h 2, and Ara h 3 are minor allergens; only 4.8% (Table II) of the subjects with peanut allergy showed IgE antibody reactivities to any of these allergens. Ara h 9, however, was recognized by 45.2% of the subjects with peanut allergy. From these data, it can be deduced that no association between the IgE reactivity to the major peanut allergens Ara h 1, 2, and 3 and the sensitization to Ara h 9 exists. The statistical analysis by the Fisher exact test did not show any statistically significant associations.

### Peach LTP may be a sensitizing allergen in peanut allergy

As demonstrated in Table II, cosensitization to peach LTP (Pru p 3, 38.1%) was higher than that to hazelnut LTP (Cor a 8, 16.7%), supporting the notion that Pru p 3 may be the primary sensitizing allergen in cases in which the classic major peanut allergens were not involved in primary sensitization to peanut. This was supported by the inhibition experiments (SPHIAa, Table III) showing that Ara h 9 does not bear all the IgE-binding epitopes to inhibit the IgE antibody reactivity to both Cor a 8 and Pru p 3, although differences were recorded from subject to subject, ranging from almost no inhibition to full inhibition (Table III).

Other sources of sensitization (Bet v 1 homologs, profilin) were also very unlikely because only 14.3% and 11.9% of the sera of subjects with peanut allergy contained specific IgE antibodies to any of the 4 Bet v 1 homologs and 5 profilins, respectively (Table II). Even the Bet v 1 homolog from peanut Ara h 8 bound IgE from only 4 of 42 (9.5%) sera of subjects with peanut allergy (Table I).

**TABLE I.** General characteristics of patients and *in vitro* diagnostics

| Strip number on IgE immuno-blot | Sex | Age (y) | Recruitment group | Clinical status | Allergy to |        | IgE                 |                    |                  |                 | Other LTPs   |                |                | Major peanut allergens |                |                |
|---------------------------------|-----|---------|-------------------|-----------------|------------|--------|---------------------|--------------------|------------------|-----------------|--------------|----------------|----------------|------------------------|----------------|----------------|
|                                 |     |         |                   |                 | Peanut*    | Peach* | Peanut (CAP, IU/mL) | Peach (CAP, IU/mL) | Peanut (pos/neg) | Peach (pos/neg) | Ara h 9 (IB) | Prp p 3 (ISAC) | Cor a 8 (ISAC) | Ara h 1 (ISAC)         | Ara h 2 (ISAC) | Ara h 3 (ISAC) |
| 1                               | F   | 27      | I                 | A               | Yes        | Yes    | 36.20               | 86.50              | +                | +               | +            | +              | +              | -                      | -              | -              |
| 2                               | M   | 23      | I                 | B               | Yes        | No     | ND                  | 14.80              | ND               | +               | +            | +              | +              | -                      | -              | -              |
| 3                               | M   | 9       | I                 | A               | Yes        | Yes    | 9.62                | 51.60              | +                | +               | +            | +              | +              | +                      | -              | -              |
| 4                               | M   | 11      | I                 | B               | Yes        | No     | 0.90                | 19.20              | +                | +               | +            | +              | -              | -                      | -              | -              |
| 5                               | F   | 44      | I                 | A               | Yes        | Yes    | 0.43                | 3.31               | +                | +               | -            | +              | -              | -                      | -              | -              |
| 6                               | M   | 17      | I                 | B               | Yes        | No     | 5.36                | 8.33               | +                | +               | +            | +              | ND             | ND                     | ND             | ND             |
| 7                               | F   | 57      | I                 | A               | Yes        | Yes    | 4.36                | 2.53               | +                | +               | -            | +              | -              | -                      | -              | -              |
| 8                               | M   | 25      | I                 | B               | Yes        | No     | 7.09                | 27.80              | +                | +               | +            | +              | -              | -                      | -              | -              |
| 9                               | F   | 28      | I                 | A               | Yes        | Yes    | 10.80               | 10.60              | +                | +               | +            | +              | -              | -                      | -              | -              |
| 10                              | M   | 15      | I                 | A               | Yes        | Yes    | 8.34                | 16.80              | +                | +               | +            | +              | +              | -                      | -              | -              |
| 11                              | M   | 35      | I                 | B               | Yes        | No     | 1.94                | 4.97               | +                | +               | -            | +              | -              | -                      | -              | -              |
| 12                              | M   | 38      | I                 | B               | Yes        | No     | 1.10                | 2.82               | +                | +               | +            | +              | -              | -                      | -              | -              |
| 13                              | F   | 19      | I                 | B               | Yes        | No     | 0.60                | 4.98               | +                | +               | -            | +              | -              | -                      | -              | -              |
| 14                              | F   | 25      | I                 | B               | Yes        | No     | 27.80               | 64.50              | +                | +               | +            | +              | ND             | ND                     | ND             | ND             |
| 15                              | F   | 41      | I                 | A               | Yes        | Yes    | 3.95                | 9.74               | +                | +               | -            | +              | +              | -                      | -              | -              |
| 16                              | F   | 30      | I                 | B               | Yes        | No     | 12.40               | 44.10              | +                | +               | +            | +              | ND             | ND                     | ND             | ND             |
| 17                              | F   | 31      | I                 | B               | Yes        | No     | 0.43                | 1.01               | +                | +               | -            | +              | -              | -                      | -              | -              |
| 18                              | F   | 39      | I                 | A               | Yes        | Yes    | 6.26                | 12.20              | +                | +               | +            | +              | +              | -                      | -              | -              |
| 19                              | M   | 12      | I                 | A               | Yes        | Yes    | 1.17                | 7.92               | +                | +               | -            | +              | -              | -                      | -              | -              |
| 20                              | F   | 13      | I                 | A               | Yes        | Yes    | 13.80               | 54.80              | +                | +               | +            | +              | ND             | ND                     | ND             | ND             |
| 21                              | F   | 32      | I                 | A               | Yes        | Yes    | 3.10                | 3.90               | +                | +               | -            | +              | -              | -                      | -              | -              |
| 22                              | F   | 25      | I                 | A               | Yes        | Yes    | 56.40               | 51.30              | +                | +               | +            | +              | +              | -                      | -              | -              |
| 23                              | M   | 20      | I                 | B               | Yes        | No     | 7.47                | 6.12               | +                | +               | -            | +              | -              | -                      | -              | -              |
| 24                              | F   | 19      | I                 | B               | Yes        | No     | 25.30               | 62.30              | +                | +               | +            | +              | +              | -                      | -              | -              |
| 32                              | M   | 22      | I                 | A               | Yes        | Yes    | 1.79                | 8.47               | +                | +               | +            | +              | -              | -                      | -              | -              |
| 25                              | F   | 21      | II                | A               | Yes        | Yes    | 3.40                | 3.66               | +                | +               | -            | -              | -              | -                      | -              | -              |
| 26                              | M   | 13      | II                | B               | Yes        | No     | <0.35               | ND                 | -                | nd              | -            | -              | -              | -                      | -              | -              |
| 27                              | F   | 44      | II                | B               | Yes        | No     | <0.35               | <0.35              | -                | -               | -            | -              | -              | -                      | -              | -              |
| 28                              | F   | 19      | II                | B               | Yes        | No     | 1.90                | <0.35              | +                | -               | -            | -              | -              | -                      | -              | -              |
| 29                              | M   | 27      | II                | B               | Yes        | No     | <0.35               | <0.35              | -                | -               | -            | -              | -              | -                      | -              | -              |
| 30                              | M   | 55      | II                | A               | Yes        | Yes    | <0.35               | 1.25               | -                | +               | -            | -              | -              | -                      | -              | -              |
| 31                              | F   | 40      | II                | B               | Yes        | No     | 0.66                | 0.51               | +                | +               | -            | -              | -              | -                      | -              | -              |
| 33                              | M   | 53      | II                | B               | Yes        | No     | <0.35               | <0.35              | -                | -               | -            | -              | -              | -                      | -              | -              |
| 34                              | F   | 16      | II                | B               | Yes        | No     | 0.95                | 1.88               | +                | +               | +            | -              | -              | -                      | -              | -              |
| 35                              | F   | 27      | II                | B               | Yes        | No     | <0.35               | <0.35              | -                | -               | -            | -              | -              | -                      | -              | -              |
| 36                              | M   | 7       | II                | B               | Yes        | No     | <0.35               | <0.35              | -                | -               | -            | -              | -              | -                      | -              | -              |
| 37                              | M   | 15      | II                | B               | Yes        | No     | 9.47                | 22.40              | +                | +               | +            | -              | -              | -                      | -              | -              |
| 38                              | M   | 16      | II                | A               | Yes        | Yes    | 5.84                | 12.50              | +                | +               | +            | -              | -              | -                      | -              | -              |
| 39                              | M   | 7       | II                | B               | Yes        | No     | 7.95                | 2.15               | +                | +               | -            | -              | -              | +                      | -              | -              |
| 40                              | F   | 56      | II                | B               | Yes        | No     | 1.13                | 0.62               | +                | +               | -            | -              | -              | -                      | -              | -              |
| 41                              | F   | 23      | II                | B               | Yes        | No     | <0.35               | <0.35              | -                | -               | -            | -              | -              | -                      | -              | -              |
| 42                              | F   | 24      | II                | B               | Yes        | No     | 1.68                | 1.43               | +                | +               | -            | -              | -              | -                      | -              | -              |
| 43                              | F   | 20      | III               | C               | No         | Yes    | <0.35               | 4.81               | -                | +               | -            | +              | -              | -                      | -              | -              |
| 44                              | F   | 39      | III               | C               | No         | Yes    | 0.78                | 9.79               | +                | +               | -            | +              | +              | -                      | -              | -              |
| 45                              | F   | 20      | III               | C               | No         | Yes    | ND                  | ND                 | ND               | ND              | +            | +              | ND             | ND                     | ND             | ND             |
| 46                              | F   | 23      | III               | C               | No         | Yes    | 4.02                | 6.16               | +                | +               | +            | +              | +              | -                      | -              | -              |
| 47                              | M   | 31      | III               | D               | No         | No     | 0.71                | 3.83               | +                | +               | +            | +              | -              | -                      | -              | -              |
| 48                              | F   | 27      | III               | D               | No         | No     | <0.35               | 30.20              | -                | +               | -            | +              | -              | -                      | -              | -              |
| 49                              | F   | 38      | III               | C               | No         | Yes    | <0.35               | <0.35              | -                | -               | -            | +              | -              | -                      | -              | -              |
| 50                              | F   | 19      | III               | C               | No         | Yes    | <0.35               | 4.83               | -                | +               | +            | +              | -              | -                      | -              | -              |
| 51                              | M   | 30      | III               | D               | No         | No     | 10.90               | 22.80              | +                | +               | -            | +              | +              | -                      | -              | -              |
| 52                              | F   | 28      | III               | C               | No         | Yes    | <0.35               | 0.59               | -                | +               | -            | +              | -              | -                      | -              | -              |
| 53                              | M   | 26      | III               | D               | No         | No     | <0.35               | 4.13               | -                | +               | -            | +              | +              | -                      | -              | -              |
| 54                              | F   | 35      | III               | C               | No         | Yes    | 4.08                | 1.22               | +                | +               | -            | +              | -              | -                      | -              | -              |
| 55                              | F   | 20      | III               | C               | No         | Yes    | 0.54                | 1.12               | +                | +               | -            | +              | -              | -                      | -              | -              |
| 56                              | F   | 41      | III               | D               | No         | No     | <0.35               | <0.35              | -                | -               | -            | +              | -              | -                      | -              | -              |
| 57                              | F   | 43      | III               | C               | No         | Yes    | 1.36                | 3.31               | +                | +               | -            | +              | -              | -                      | -              | -              |
| 58                              | M   | 26      | III               | C               | No         | Yes    | 4.26                | 4.92               | +                | +               | -            | +              | -              | -                      | -              | -              |
| 59                              | F   | 38      | III               | C               | No         | Yes    | 10.90               | 27.70              | +                | +               | +            | +              | +              | -                      | -              | -              |
| 60                              | M   | 22      | III               | C               | No         | Yes    | <0.35               | 0.45               | -                | +               | -            | +              | -              | -                      | -              | -              |
| 61                              | M   | 30      | III               | D               | No         | No     | 13.00               | 22.40              | +                | +               | -            | +              | -              | -                      | -              | -              |
| 62                              | M   | 73      | III               | C               | No         | Yes    | <0.35               | 3.41               | -                | +               | -            | +              | -              | -                      | -              | -              |
| 63                              | F   | 36      | III               | C               | No         | Yes    | 43.70               | 89.30              | +                | +               | -            | +              | ND             | ND                     | ND             | ND             |
| 64                              | F   | 29      | III               | D               | No         | No     | 1.77                | ND                 | +                | ND              | -            | +              | +              | -                      | -              | -              |
| 65                              | F   | 22      | III               | D               | No         | No     | 22.10               | 37.30              | +                | +               | +            | +              | +              | -                      | -              | -              |
| 66                              | F   | 20      | III               | D               | No         | No     | 1.27                | 7.01               | +                | +               | -            | +              | -              | -                      | -              | -              |

Amounts of specific IgE antibodies to peanut extract (CAP-PEIA), peach extract (CAP-PEIA), Pru p 3 (ISAC), Ara h 9 (immunoblot analysis), skin prick testing with peanut and peach, the individual symptoms (A, asthma; AE, angioedema; AP, abdominal pain; C, conjunctivitis; D, dyspnea; Dp, disphonia; Dr, diarrhea; E, eczema; LE, laryngeal edema; N, nausea; OAS, oral allergy syndrome; P, pruritus; R, rhinitis; U, urticaria; Vo, vomiting). Strip numbers refer to Fig 1. F, Female; IB, immunoblotting; M, male; ND, not determined; neg, negative; pos, positive; SPT, skin prick test.

TABLE I. (Continued)

| Bet v 1 homologs  |                   |                   |                   | Profilins         |                   |                   |                   |                    | SPT               |                  |                       |                      |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|--------------------|-------------------|------------------|-----------------------|----------------------|
| Ara h 8<br>(ISAC) | Cor a 1<br>(ISAC) | Mal d 1<br>(ISAC) | Pru p 1<br>(ISAC) | Bet v 2<br>(ISAC) | Hev b 8<br>(ISAC) | Mer a 1<br>(ISAC) | Ole e 2<br>(ISAC) | Phi p 12<br>(ISAC) | Peanut<br>extract | Peach<br>extract | Symptoms<br>to peanut | Symptoms<br>to peach |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                | U                     | U, AE                |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               | U, AE                 |                      |
| -                 | -                 | -                 | -                 | +                 | +                 | +                 | +                 | +                  | +                 | +                | OAS, E                | OAS                  |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               | LE                    |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                | AP, N                 | AE                   |
| ND                | ND                | ND                | ND                | ND                | ND                | ND                | ND                | ND                 | +                 | ND               | AE                    |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | -                 | -                | A                     | A                    |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                | AE                    |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                | OAS                   | AP, Dr               |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | -                | OAS                   | OAS, P               |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | -                 | +                | OAS                   |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                | OAS                   |                      |
| -                 | -                 | -                 | +                 | -                 | -                 | -                 | -                 | -                  | +                 | +                | AP                    |                      |
| ND                | ND                | ND                | ND                | ND                | ND                | ND                | ND                | ND                 | +                 | +                | OAS                   |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               | U                     | OAS                  |
| ND                | ND                | ND                | ND                | ND                | ND                | ND                | ND                | ND                 | +                 | +                | D                     |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                | U, AE                 |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               | N, Vo                 | N, Vo                |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                | U, R                  | OAS, P, C            |
| ND                | ND                | ND                | ND                | ND                | ND                | ND                | ND                | ND                 | ND                | ND               | AP                    | N, Vo                |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                | OAS                   | OAS                  |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | -                | AE, AP, N             | U                    |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | -                | U, AE                 |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               | AE                    |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | ND               | OAS                   | OAS                  |
| +                 | +                 | +                 | +                 | +                 | +                 | +                 | +                 | +                  | +                 | -                | AE                    | OAS                  |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               | U                     |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               | AE, OAS               |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               | Dr                    |                      |
| -                 | -                 | -                 | -                 | +                 | +                 | +                 | -                 | -                  | ND                | ND               | AE                    |                      |
| -                 | +                 | +                 | +                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               | AE                    | OAS                  |
| -                 | -                 | -                 | -                 | +                 | +                 | +                 | +                 | +                  | ND                | ND               | OAS                   |                      |
| -                 | -                 | -                 | -                 | -                 | +                 | +                 | -                 | -                  | ND                | ND               | D                     |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | -                | AE, OAS               |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                | Dr                    |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               | OAS                   |                      |
| +                 | +                 | +                 | +                 | -                 | -                 | -                 | -                 | -                  | +                 | -                | OAS                   |                      |
| +                 | +                 | +                 | +                 | -                 | -                 | -                 | -                 | -                  | +                 | -                | OAS                   | OAS                  |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | -                | U, AE, D              |                      |
| +                 | +                 | +                 | +                 | -                 | -                 | -                 | -                 | -                  | +                 | -                | OAS, D                |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               | AE, OAS               |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | -                 | -                | A                     |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | -                 | +                |                       | U, AE, OAS           |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                | OAS                   |                      |
| ND                | ND                | ND                | ND                | ND                | ND                | ND                | ND                | ND                 | ND                | ND               |                       | OAS                  |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                |                       | OAS                  |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               |                       |                      |
| +                 | +                 | +                 | +                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               |                       |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | -                 | -                |                       | OAS                  |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               |                       | AE, Dr, Vo           |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               |                       |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               |                       | OAS                  |
| -                 | -                 | -                 | -                 | +                 | +                 | +                 | +                 | +                  | ND                | ND               |                       | OAS                  |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                |                       | AE, Dp               |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               |                       |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               |                       | OAS                  |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | -                |                       | OAS, AP              |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               |                       | OAS, P               |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | -                 | +                |                       | OAS                  |
| -                 | -                 | -                 | +                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               |                       |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               |                       | AE                   |
| ND                | ND                | ND                | ND                | ND                | ND                | ND                | ND                | ND                 | ND                | ND               |                       | U, OAS, Dr           |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               |                       |                      |
| -                 | +                 | +                 | +                 | -                 | -                 | -                 | -                 | -                  | ND                | ND               |                       |                      |
| -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                 | -                  | +                 | +                |                       |                      |

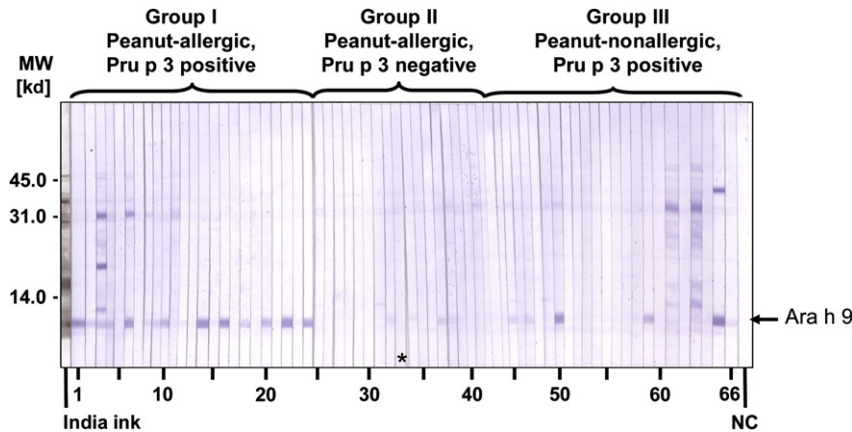


**FIG 1.** Immunoblot analysis of IgE antibody reactivities of subjects' sera to Ara h 9-enriched peanut extract. No. 1-66, Patients' sera; India ink, protein staining; MW, molecular weight; NC, negative control. One strip (\*subject 32) belongs to group I.

**TABLE II.** Association of frequencies of sensitizations in percent of subjects with peanut and peach allergy to Ara h 9, Pru p 3, Cor a 8, the major peanut allergens Ara h 1, Ara h 2, and Ara h 3, Bet v 1 homologs, and profilins

| Description                    | N  | Specific IgE reactivities to |         |                     |                     |                               |                     |
|--------------------------------|----|------------------------------|---------|---------------------|---------------------|-------------------------------|---------------------|
|                                |    | Ara h 9                      | Pru p 3 | Ara h 9 and Pru p 3 | Ara h 9 and Cor a 8 | Ara h 1 or Ara h 2 or Ara h 3 | Any Bet v 1 homolog |
| Peanut and peach allergy (A)   | 16 | 56.3                         | 81.3    | 50.0                | 33.3                | 6.7                           | 20.0                |
| Only peanut allergy (B)        | 26 | 38.5                         | 46.2    | 30.8                | 8.7                 | 4.3                           | 13.0                |
| Only peach allergy (C)         | 15 | 26.7                         | 100.0   | 26.7                | 15.4                | 0.0                           | 7.7                 |
| No peanut or peach allergy (D) | 9  | 22.2                         | 100.0   | 22.2                | 11.1                | 0.0                           | 11.1                |
| Peanut allergy (A + B)         | 42 | 45.2                         | 59.5    | 38.1                | 16.7                | 4.8                           | 14.3                |
| Peach allergy (B + C)          | 31 | 45.2                         | 87.1    | 38.7                | 12.9                | 3.2                           | 9.7                 |

## Two Ara h 9 isoforms—Ara h 9.0101 and Ara h 9.0201—were cloned and expressed

For the recombinant production of peanut LTP, total RNA was isolated from peanut flour for cDNA generation. Because of the unknown sequence of peanut LTP, cDNAs of LTPs from cherry, peach, lentil, strawberry, and hazelnut were aligned to identify conserved regions. A sequence stretch with high sequence identity in the 3' region was found (Fig E1). With this information, it was possible to amplify the 3' end of the peanut LTP. Because the 5' rapid amplification of cDNA ends failed, a nucleotide BLAST search against the expressed sequence tags database was performed with the obtained 3' end. One hundred eighty-nine full-length sequences with sequence identities of 100% to the applied 3' region were found. To obtain unbiased LTP gene sequences, the 5' untranslated region was used to design a forward primer. Full length cDNAs of 2 Ara h 9 isoforms were obtained in this way. The SignalP 3.0 software<sup>27</sup> (<http://www.cbs.dtu.dk/services/SignalP>) (provided by Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark) predicted a 24-amino acid signal peptide in both isoforms, which was confirmed by N-terminal sequencing of natural peanut LTP (Fig E4) starting at amino acid 25. The 2 LTP isoforms sharing 90% sequence identity were named Ara h 9.0101 and Ara h 9.0201 and have been officially accepted by the IUIS Allergen Nomenclature Sub-committee as new peanut allergens. The sequences were entered into the GenBank with the accession numbers EU159429 and EU161278. With the exception of the first amino acid, the recombinant peanut LTPs (see below; Fig E4) were in agreement

with the amino acid sequences of natural LTP obtained by N-terminal sequencing.

## Recombinant forms of 2 Ara h 9 isoforms are immunologically reactive

To test the recombinant peanut LTP variants for their immunologic properties, sera from subjects with IgE antibodies to natural peanut LTP were used to test the IgE antibody reactivity of rAra h 9.0101 and rAra h 9.0201 by immunoblot analysis (Fig 2). As shown in Fig 2, patients' sera reactive to natural Ara h 9 (Fig 1) bind to both recombinant isoforms with the exception of one serum (#50 in Fig 1; strip 6 in Fig 2). Protein sequencing of the 21-kd and the 10.5-kd IgE-reactive bands confirmed the N-terminus of Ara h 9.0201, suggesting that the former one is a dimer. One additional serum (#60 in Fig 1; strip 13 in Fig 2) served as a control because it did not react with natural and recombinant Ara h 9. These results demonstrate that both rAra h 9 isoforms were IgE-reactive. Inhibition experiments with rAra h 9.02 and acidic peanut extract with enriched nAra h 9 as target (data not shown) revealed in a semiquantitative manner the immunologic similarity of rAra h 9.02 and nAra h 9. Additional potential isoforms and variants of Ara h 9 were identified by bioinformatics (Fig E4).

## DISCUSSION

The aim of this study was to identify serologically reactive LTP in peanut extract. The first evidence to the existence of allergenic

TABLE III. SPHIAa using the ISAC microarray for IgE detection

| Serum | rCor a 8 on ISAC             |                       |        |                            |        | rPru p 3 on IS               |                       |        |                            |        |
|-------|------------------------------|-----------------------|--------|----------------------------|--------|------------------------------|-----------------------|--------|----------------------------|--------|
|       | No inhibitor, IgE<br>(IU/mL) | nPru p 3 as inhibitor |        | rAra h 9.0201 as inhibitor |        | No inhibitor, IgE<br>(IU/mL) | nPru p 3 as inhibitor |        | rAra h 9.0201 as inhibitor |        |
|       |                              | Spec. IgE<br>(IU/mL)  | % inh  | Spec. IgE<br>(IU/mL)       | % inh  |                              | Spec. IgE<br>(IU/mL)  | % inh  | Spec. IgE<br>(IU/mL)       | % inh  |
| S-001 | 11.6                         | 2.4                   | 79.3%  | 12.7                       | -9.7%  | 41.5                         | 0.0                   | 100.0% | 20.1                       | 51.6%  |
| S-002 | 6.4                          | 0.0                   | 100.0% | 0.0                        | 100.0% | 31.4                         | 0.0                   | 100.0% | 16.3                       | 48.2%  |
| S-003 | 11.0                         | 0.0                   | 100.0% | 0.6                        | 94.6%  | 27.1                         | 0.0                   | 100.0% | 11.0                       | 59.5%  |
| S-004 | 0.0                          | 0.0                   | NA     | 0.0                        | NA     | 33.6                         | 0.0                   | 100.0% | 36.2                       | -7.6%  |
| S-005 | 1.0                          | 0.0                   | 100.0% | 0.0                        | 100.0% | 7.4                          | 0.0                   | 100.0% | 1.5                        | 80.2%  |
| S-006 | 9.2                          | 0.0                   | 100.0% | 6.6                        | 28.1%  | 20.1                         | 0.0                   | 100.0% | 12.7                       | 37.0%  |
| S-007 | 0.0                          | 0.0                   | NA     | 0.0                        | NA     | 3.9                          | 0.0                   | 100.0% | 0.0                        | 100.0% |
| S-008 | 0.00                         | 0.00                  | NA     | 0.00                       | NA     | 0.23                         | 0.00                  | 100.0% | 0.06                       | 73.9%  |
| S-009 | 0.0                          | 0.0                   | NA     | 0.0                        | NA     | 7.7                          | 0.0                   | 100.0% | 7.0                        | 9.8%   |
| S-010 | 4.9                          | 0.0                   | 100.0% | 4.2                        | 13.2%  | 6.0                          | 0.0                   | 100.0% | 4.3                        | 28.2%  |

% inh, Inhibition in percent; NA, not applicable; spec. IgE, specific IgE.

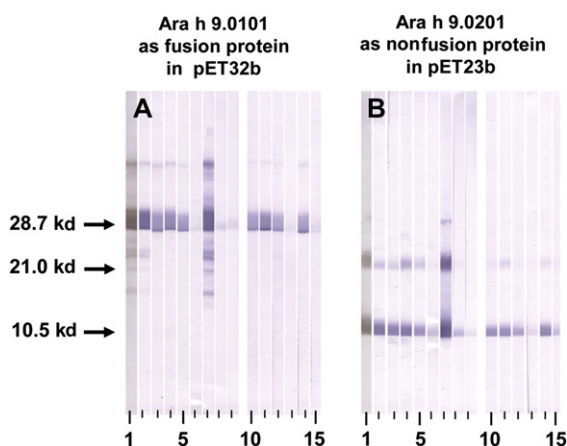


FIG 2. Immunoblot analysis of recombinant Ara h 9.0101 (A) as thioredoxin fusion protein and Ara h 9.0201 (B). Lane 1, India ink staining; lanes 2-6 and 10-14, patients' sera reactive with natural Ara h 9 (numbers in brackets correspond to the lane numbers of Fig 1: lanes 2 [6], 3 [16], 4 [22], 5 [24], 6 [50], 10 [10], 11 [14], 12 [20], 13 [60], 14 [65]); lane 7, anti-His-tag antibody-AP; lanes 8 and 15, negative controls, anti-human IgE-AP; lane 9, negative control, anti-mouse IgG + IgM-AP.

LTP in peanuts was published by Asero et al.<sup>18</sup> It was necessary to deviate from standard extraction protocols to optimize the yield of LTP by choosing acidic extraction conditions.

Probing peanut extract by Western blotting with a potentially cross-reacting rabbit anti-hazelnut LTP (Cor a 8) antiserum, no reactivity was detectable in our hands. However, we observed in corresponding experiments on hazelnut that the natural Cor a 8 content in hazelnut extracts prepared under acidic conditions was dramatically increased (Fig E3, D and E). Peanut extract prepared under acidic conditions contained faint bands of Ara h 1, Ara h 2, Ara h 3/4, and Ara h 6, in contrast with intense bands of Ara h 8 and the putative peanut LTP.<sup>28</sup>

After further enrichment of proteins with molecular weights below 20 kd, a band at 9 kd was detectable with a polyclonal anti-Cor a 8 antiserum. N-terminal sequencing confirmed the identity of this protein as LTP. The majority (16/25; 64.0%) of subjects' sera from group I (peanut-allergic, Pru p 3 positive) reacted with this band. When the IgE reactivities of all subjects with peanut allergy were considered, less than 50% of sera

from these subjects were detectable, categorizing Ara h 9 as a minor allergen.<sup>29,30</sup>

The relevance of Ara h 9 as an allergen depends on the purpose of the analysis. Measuring IgE reactivities to Ara h 9 seems not to be necessary when the aim is to increase the frequency of the detection. None of the patients who had peanut allergy and were negative to peanut extract by CAP-FEIA showed IgE reactivities to Ara h 9. However, because the classic major peanut allergens Ara h 1, Ara h 2, and Ara h 3 were minor allergens and did not contribute much to the detection of sensitizations to peanut in the population of our study, it seems prudent to include Ara h 9 in panels of single peanut allergens for component-resolved diagnosis. Only 4.8% of the subjects with peanut allergy had IgE antibody reactivities to the classic major peanut allergens. Ara h 9, however, was recognized by a little less than 50% of the subjects with peanut allergy. The observed rates of cosensitizations and the inhibition studies performed on peach LTP (Pru p 3) and hazelnut LTP (Cor a 8) support the notion that Pru p 3 or Ara h 9 may be the primary sensitizing allergens in cases in which the classic major peanut allergens were not responsible for primary sensitization to peanuts. For that, the application of Ara h 9 for component-resolved diagnosis is not only relevant as important diagnostic tool but also required to get more detailed information on the pathway of sensitization to develop preventive strategies in food allergy.

To obtain peanut LTP as a recombinant molecule, the original strategy was to clone it by PCR. Because the well established strategy using 5' rapid amplification of cDNA ends-PCR failed, we used information from the National Center of Biotechnology Information, Bethesda, Maryland expressed sequence tags database to obtain the LTP gene sequence. Pursuing this strategy, 2 isoforms of peanut LTP were cloned. The translated LTP sequences were analyzed *in silico* for signal sequences identifying a 24-amino acid signal peptide. This is in accordance with the N-terminus of natural peanut LTP. The 2 peanut LTP isoforms were expressed in *E coli* as mature proteins. Both peanut LTPs show IgE reactivity and were accepted from the IUIS Allergen Nomenclature Sub-committee as the new peanut allergens Ara h 9.0101 and Ara h 9.0201. Thus, the prerequisite for evaluating the clinical relevance of peanut

LTPs in clinical studies is given. The selection of the sera used in this study was biased toward sera with possible reactivities to peanut LTP. The study clearly established peanut LTP as a relevant allergen, which defines better the overall LTP IgE reactivity of subjects with allergy.

In conclusion, Ara h 9 is a new member of the LTP allergen family that seems to play a role in allergic sensitization and might be a trigger of symptoms. This seems to affect populations in which peanuts are not largely consumed, whereas reactivity to fresh fruit caused by LTP sensitization is frequently recorded.

**Clinical implications: The study clearly establishes peanut LTP (Ara h 9) as a relevant allergen, which should be included in allergen panels for component-resolved diagnosis studies because not all the peanut-sensitized subjects react to the classic major peanut allergens.**

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## METHODS

### ISAC technology

Immuno solid-phase allergen chip technology is the detection of specific IgE to an array of single allergen molecules spotted in triplicate on a microarray solid surface.<sup>E1</sup> Two different ISAC microarrays were used during this study. The ISAC89 was used for patients' initial selection. The same serum samples were retested with the ISAC103 chip carrying several new allergens relevant for this study, including the peanut allergens Ara h 1, Ara h 2, and Ara h 3. Briefly, 20  $\mu$ L serum was incubated in a humid chamber for 2 hours. Slides were washed. Fluorescein-labeled anti-IgE antibody was added. The slides were incubated for 1 hour, washed again, and dried. Fluorescence was detected by a laser scanner. Images were processed and results analyzed by the ISAC software (VBC-Genomics, Vienna, Austria). Values were expressed as kU/L; however, the results of all IgE measurements performed by ISAC were categorized as positive or negative (Table I). ISAC testing did not show any unspecific binding.

### Bioinformatics identified potential additional Ara h 9 isoforms and variants

Restricting the search to sequences from *Arachis hypogaea*, the query of the expressed sequence tags database (<http://blast.ncbi.nlm.nih.gov>) revealed 189 full-length sequences with sequence identities of 100% with the primer LTP\_hom\_for. Sequences that translated in the same amino acid sequences of Ara h 9.0101 and Ara h 9.0201 were found 69 times and 102 times,

respectively. Eighteen additional isoforms were found (termed Ara h LTP 03 to Ara h LTP 20). The alignment is depicted in Fig E4. The sequence identities of the mature forms among Ara h 9.0101, Ara h 9.0201, and Ara h LTP 03 to Ara h LTP 17 ranged from 88.3% to 99.2%. Complete sequence identity among Ara h LTP 05, Ara h LTP 06, and Ara h LTP 07 was a result of sequence differences among the signal peptides rather than the mature (N-terminally truncated) LTPs. The sequences Ara h LTP 18, Ara h LTP 19, and Ara h LTP 20 may even represent a different allergenic LTP because the maximal sequence identities with Ara h 9.0101, Ara h 9.0201, and Ara h LTP 03 to Ara h LTP 17 reached only 50.0% and thus did not fulfill the definition of an allergen isoform, which requires sequence identities of more than 67%.<sup>E2,E3</sup> Thus, in the future, more detailed proteomic and genomic analyses should be performed (eg, two-dimensional polyacrylamide gel electrophoresis [2D-PAGE], trypsin mass fingerprinting) to authenticate these isoforms and study the clinical relevance of these isoforms.

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|                     |                                                               |     |
|---------------------|---------------------------------------------------------------|-----|
| Pru_av_3_AF221501   | ATGGCTTGCTCTGCAATGACTAAGCTTGCTTTGGTGGTGGCCTTGTCATGGTTGTGAGC   | 60  |
| Pru_p_3_AY792996    | .....A.....C.....                                             | 60  |
| Fra_a_LTP_DQ066732  | .....G...TG.....T.CTCT.A.T.....T..G..AGC.                     | 57  |
| Cor_a_8_AF329829    | -----A.GG.T.GCC-T...T.G.TA.GC.C...CCT.....A.G...GC.           | 51  |
| Lens_c_LTP_AY793556 | -----ATGG.AAG..GC.TG...T.A..A.GT..T...TTTGG..ATGTGTA.GA.AGTT  | 54  |
| Pru_av_3_AF221501   | GTGCCCATTGCTCAAGCC---CTAACATGTGGCCAAGTGTCCAGCAACCTTGACCATGCG  | 117 |
| Pru_p_3_AY792996    | .....A.....G.....                                             | 117 |
| Fra_a_LTP_DQ066732  | .C...G.C..C.....GCCA.C..T..C.....A..CA...G.G...C...           | 117 |
| Cor_a_8_AF329829    | .CA...G...C.GG..GTCC..G...CCCA..GA.AAAAG...CA.G.....          | 111 |
| Lens_c_LTP_AY793556 | .C...T..G..AG...GTGCAA.CT.C.....AGCT..AA.TG.TG....AGT.....    | 114 |
| Pru_av_3_AF221501   | ATAGCCTACGTGAGAGGCGGTGGAGCTGTGCCTCCAGCTTGCTGCAACGGCATTAGGAAC  | 177 |
| Pru_p_3_AY792996    | ...C.....                                                     | 177 |
| Fra_a_LTP_DQ066732  | T.CAA.....C.AGA.T...C...C...G.C.....A..AG...C...              | 177 |
| Cor_a_8_AF329829    | G.GCT...C...AGAA...C..C.T.C.T...CT.....G...G.C...GCT          | 171 |
| Lens_c_LTP_AY793556 | C.TA.G...TC.T.C...T...CCT.G.CCTT.A...CAA.....TGA..AG.G.A...G  | 174 |
| Pru_av_3_AF221501   | ATCAACAACCTTGGCAAAGACCACCGCTGACCGCCAGACCGCTTGCAACTGCCTGAAACAG | 237 |
| Pru_p_3_AY792996    | G.....G.....C.....G.....                                      | 237 |
| Fra_a_LTP_DQ066732  | C.....G.A...C.....CAAA.....T.....GAGT                         | 237 |
| Cor_a_8_AF329829    | G.A...G..GCCT.C.G.....GT.C.....T.....T...G.T                  | 231 |
| Lens_c_LTP_AY793556 | C.TCTTGCTGCC..C..C.....GC...T..T...G.T..C..T...A.....TCA      | 234 |
| Pru_av_3_AF221501   | CTTTCGGCCAGCGTCCCGGAGTCAATGCTAACAATGCCGCAGCGCTTCCCGGCAAGTGT   | 297 |
| Pru_p_3_AY792996    | .....CC.....                                                  | 297 |
| Fra_a_LTP_DQ066732  | GCAG.T.GA...A..AAG..C.T..C.....CTC..A..C.GT.....              | 297 |
| Cor_a_8_AF329829    | ACAG..AAAG..A..G.T..CC...CC...TCT...T..T.GC..C.....           | 291 |
| Lens_c_LTP_AY793556 | GCAG..AGTTCTA.TA.TAA.T.G...A.....T..T..T..C..T...A...         | 294 |
| LTP_hom_for         | CATTTCCTTACAAGATCAGCACCTC                                     |     |
| Pru_av_3_AF221501   | GGAGTTAA TGTTTCCTTACAAGATCAGCCCTCCACCAACTGCGCCACCGTGAAGTGA    | 354 |
| Pru_p_3_AY792996    | .....G.A.....T...G.....                                       | 354 |
| Fra_a_LTP_DQ066732  | .T..C..C.....C.....A.....AA..AT.....A...                      | 354 |
| Cor_a_8_AF329829    | .T..C..CA.....AA..A.....                                      | 348 |
| Lens_c_LTP_AY793556 | .T..C..CA.....TA.....TAAT..A..T...A..                         | 351 |

**FIG E1.** Alignment of cDNAs of LTPs from cherry (AF221501), peach (AY792996), strawberry (DQ066732), hazelnut (AF329829), and lentil (AY793556). The lentil sequence representing positions 303 to 326 (except position 321) was selected as forward primer LTP\_hom\_for. The part of the DNA sequences revealing a high sequence identity were used to design a primer (indicated by gray shading).

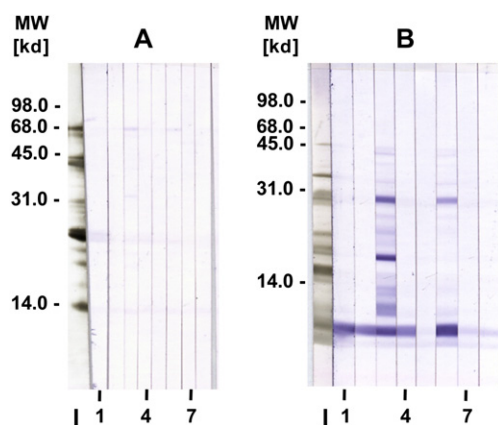


**FIG E2.** IgE reactivity to basic (A) and acidic peanut extract, fraction below 20 kD (B). Lane 1, Protein staining by India ink; lanes 1 to 8, patients' sera of subjects with peanut allergy and specific IgE antibodies to Pru p 3 (the order of patients' sera is identical with that of Fig 1). MW, Molecular weight.

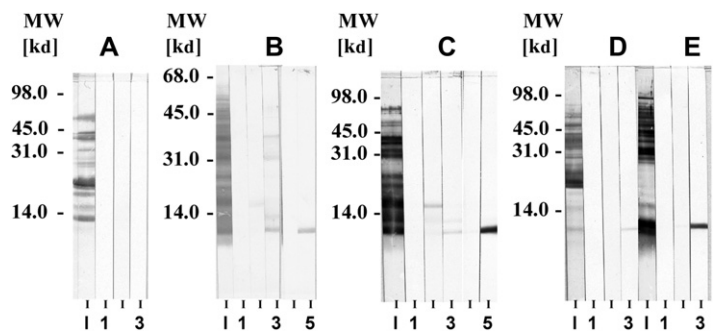


**FIG E3.** Optimizing extraction conditions of peanut LTP. Reactivity of rabbit anti-Cor a 8 antiserum against basic peanut extract (**A**), acidic peanut extract (**B**), acidic peanut fraction below 20 kD (**C**), and for comparison, basic (**D**) and acidic hazelnut extract (**E**). *Lane 1*, protein staining by India ink; *lane 2*, buffer control; *lane 3*, rabbit anti-Cor a 8; only in *B* and *C*, *lane 4*, human serum control; *lane 5*, IgE-reactive patient serum to Ara h 9. *MW*, Molecular weight.

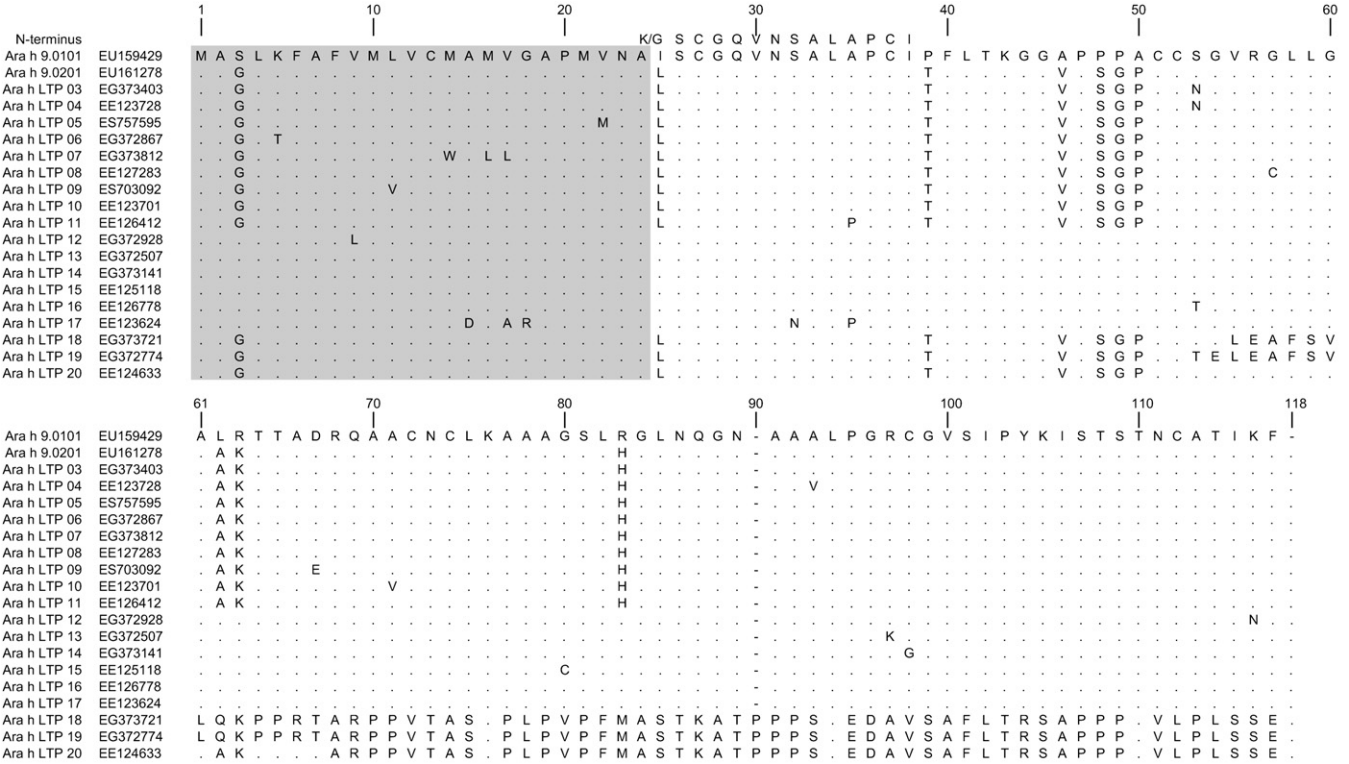


**FIG E4.** Amino acid sequence alignment of Ara h 9.0101 and Ara h 9.0201 with the N-terminus of natural Ara h 9 and amino acid sequences of 18 additional isoforms and variants. The signal peptides are shaded in gray.