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## Peanut allergens

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### Abstract

Peanut allergens have the potential to negatively impact on the health and quality of life of millions of consumers worldwide. The seeds of the peanut plant *Arachis hypogaea* contain an array of allergens that are able to induce the production of specific IgE antibodies in predisposed individuals. A lot of effort has been focused on obtaining the sequences and structures of these allergens due to the high health risk they represent. At present, 16 proteins present in peanuts are officially recognized as allergens. Research has also focused on their in-depth immunological characterization as well as on the design of modified hypoallergenic derivatives for potential use in clinical studies and the formulation of strategies for immunotherapy. Detailed research protocols are available for the purification of natural allergens as well as their recombinant production in bacterial, yeast, insect, and algal cells. Purified allergen molecules are now routinely used in diagnostic multiplex protein arrays for the detection of the presence of allergen-specific IgE. This review gives an overview on the wealth of knowledge that is available on individual peanut allergens.

### Keywords

Peanut allergens; Peanut allergy; Natural allergens; Recombinant allergens; Structural biology; Food processing; Component-resolved diagnosis; Allergy immunotherapy; Clinical studies

## 1 The role of peanut proteins in peanut allergy

Peanut allergy is one of the most severe food allergies which usually is not outgrown. Symptoms can be triggered by tiny amounts of allergens and even manifest as severe anaphylaxis. A survey made in the USA registered an increase of the prevalence of peanut allergy among children from a rate of 0.4% in 1997 to 1.4% in 2008 (Sicherer et al., 2010). This phenomenon was confirmed in the UK where an increase of the prevalence of peanut allergy was also registered (Venter et al., 2010).

The pattern of sensitization to peanut allergens varies among populations in different geographical regions (Vereda et al., 2011). The major peanut allergens Ara h 1, Ara h 2, and Ara h 3 are the main elicitors of allergic reactions in the USA and are often associated with

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severe symptoms. Spanish patients recognized these peanut allergens less frequently and were more often sensitized to the lipid transfer protein Ara h 9. Swedish patients detected Ara h 1 to 3 more frequently than Spanish patients but had the highest sensitization rate to Ara h 8, a cross-reactive homologue of the major birch pollen allergen Bet v 1. In a study involving peanut allergic subjects from 11 European countries sensitized to Ara h 1, Ara h 2 and Ara h 3 since childhood, Ara h 2 was identified as the sole major allergen (Ballmer-Weber et al., 2015). Geographical differences were observed for Ara h 8 and Ara h 9, which were major allergens for Central/Western and Southern Europeans, respectively. In a study of peanut allergic patients from the Netherlands, the most frequently recognized allergen was also Ara h 2 (Koppelman et al., 2004).

Peanut profilin, Ara h 5, is another allergen responsible for pollen-associated peanut allergy. IgE reactivity to Ara h 5 was shown in a Swedish cohort of peanut allergic individuals to be associated with that of the profilins from grass and birch pollen, Phl p 12 and Bet v 2, respectively (Cabanos et al., 2010a). In a study of individuals from the Swedish BAMSE birth cohort, children sensitized to both peanut and birch pollen were less likely to report symptoms to peanut than children sensitized to peanut but not to birch pollen at 8 years (Asaranoj et al., 2010). Sensitization to peanut oleosins was associated with severe systemic reactions (Schwager et al., 2017). No data are available on the prevalence or allergenic activity of Ara h 7. More studies are also needed to address the immunological properties of Ara h 12 and Ara h 13, the peanut defensins, which were recently found to be reactive with IgE from patients with severe peanut allergy (Petersen et al., 2015).

## 2 Biological functions of peanut allergens

Seed storage proteins are present as one or more groups of proteins in high amounts in seeds to provide a store of amino acids for use during germination and seed growth. Ara h 1 and Ara h 3 are bicupin seed storage proteins. They belong to the cupin superfamily, a functionally highly diverse protein superfamily (Dunwell et al., 2004) which contains at present 61 member families. In legumes, such as the peanut, the globulin type seed storage proteins are present in two forms, the 7S trimeric vicilins (e.g. Ara h 1) and the 11S hexameric legumins (e.g. Ara h 3). Experiments performed by Viquez and colleagues revealed that Ara h 1 had trypsin inhibitory activity indicating that the protein might play a role in plant defense against insects (Viquez et al., 2003). Interestingly, the peptide that is cleaved off at the N-terminus to yield mature Ara h 1 contains six cysteine residues that might stabilize its structure against digestive denaturation (Wichers et al., 2004). The peptide resembles a class of antifungal oligopeptides from plant seeds such as Rs-AFP2, a defensin isolated from radish seeds (Terras et al., 1995).

Ara h 2, Ara h 6, and Ara h 7 are 2S albumin seed storage proteins (Mylne et al., 2014) which are members of the prolamin superfamily (Shewry and Tatham, 1990). Non-specific lipid transfer proteins (nsLTPs) form another family of the prolamin superfamily. They are present as type 1 (~9 kDa) and type 2 nsLTPs (~7 kDa) in plants and involved in stabilization of membranes, cell wall organization, signal transduction, and plant growth and development as well as in resistance to biotic and abiotic stress (Liu et al., 2015). Ara h 9 and Ara h 17 are type 1 nsLTPs while Ara h 16 is a type 2 nsLTP.

Plants contain actin-binding proteins which regulate the supramolecular organization and function of the actin cytoskeleton, including the monomer-binding profilins (McCurdy et al., 2001). Profilins regulate cytoskeletal dynamics and membrane trafficking. The peanut allergen Ara h 5 is a member of the profilin family. The major birch pollen allergen Bet v 1 is the founding member of the Bet v 1 family of proteins. Bet v 1 isoforms show an individual, highly specific binding behavior for differently glycosylated flavonoids, the physiological ligands of Bet v 1 (Seutter von Loetzen et al., 2015). Isoform and ligand mixtures have been suggested to act as fingerprints of the pollen from distinct trees and thus to play an important role in recognition processes during pollination. Ara h 8, the Bet v 1 homologous allergen from peanut, was shown to bind the isoflavones quercetin and apigenin as well as resveratrol with high avidity (Hurlburt et al., 2013).

Lipids are stored in oil seeds in specialized intracellular structures called oil bodies which are involved in various aspects of lipid and energy metabolism. They consist of a core of neutral lipids surrounded by proteins embedded in a phospholipid monolayer (Jolivet et al., 2013). Oleosins, amphiphilic structural proteins, are the most abundant oil body proteins. Ara h 10, Ara h 11, Ara h 14, and Ara h 15 are the peanut oleosins.

Plant defensins are small, cysteine-rich peptides that possess biological activity towards a broad range of organisms, their activity being primarily directed against fungi (Vriens et al., 2014). Ara h 12 and Ara h 13 are allergenic peanut defensins. The antimicrobial activity of the amphiphilic peanut defensins Ara h 12 and Ara h 13 is solely antifungal (Petersen et al., 2015). The peanut defensins showed inhibitory effects on mold strains of the genera *Cladosporium* and *Alternaria*.

### 3 Sequences of peanut allergens

To date, the WHO/IUIS Allergen Nomenclature Sub-Committee (<http://www.allergen.org>), the only body of experts authorized to assign official allergen designations, recognizes 16 peanut allergens (Table 1). The allergen Ara h 4 was renamed Ara h 3.02 and the number 4 is not available for future peanut allergen designations to avoid confusions with the already existing literature (Radauer et al., 2014).

#### 3.1 Cupins: Ara h 1, Ara h 3

Ara h 1 is a bicupin storage protein of the vicilin type. The cDNA sequences of two Ara h 1 encoding clones, 41B and P17, were published in 1995 (Burks et al., 1995). Both clones showed a sequence identity of greater than 97% and encoded proteins of around 68 kDa. Both proteins have an N-terminal 25 amino acid residue signal peptide and a single glycosylation site (NAS) at amino acid positions 521–523. A genomic Ara h 1 clone, capable of giving rise to the mRNA for the cDNA of clone 41B, consisted of four exons and three introns (Viquez et al., 2003). Its open reading frame encoded a protein of 626 residues. The first report of an N-terminal sequence of mature Ara h 1 indicated that, depending on the length of the isoallergen, 78 or 84 residues in total are cleaved off at the N-terminus during post-translational processing of Ara h 1 (de Jong et al., 1998). In SDS-PAGE, the two Ara h 1 isoforms (P43237 and P43238) appear as two closely spaced bands at 69 and 66 kDa (Wilson and Tan-Wilson, 2015). These sizes are consistent with the removal of a 25-residue

signal peptide as well as the removal of an N-terminal propeptide. Ara h 1 is translated as a pre-pro-protein. The signal peptide directs the nascent protein to the storage vacuole where the propeptide is cleaved off to yield the mature Ara h 1 found in peanuts (Hurlburt et al., 2014). The cleaved-off N-terminal propeptide contains three allergenic epitopes, of which two are major (Burks et al., 1997). The Ara h 1 monomer, which forms stable trimers held together by non-covalent interactions, occurs in peanuts as larger oligomers (van Bostel et al., 2006).

The bicupin storage protein Ara h 3 was originally identified as a 14 kDa peanut protein by Eigenmann and coworkers (Eigenmann et al., 1996). Its N-terminal sequence was determined (Burks et al., 1998) and used to design degenerate oligonucleotides for screening a peanut cDNA library (Rabjohn et al., 1999). The open reading frame of the Ara h 3 cDNA identified in this screen coded for a protein of around 60 kDa. The 14 kDa protein appeared to be an N-terminal breakdown product of the larger allergen. A genomic clone encoding Ara h 3, AF10854, revealed the presence of four exons. The deduced protein of 538 amino acid residues has a calculated molecular mass of 61.7 kDa. Ara h 3 has a leader peptide of 20 amino acid residues that is important for protein translocation to the storage vacuole. The deduced amino acid sequence showed 93% and 91% identity with the peanut allergens Ara h 3 (Rabjohn et al., 1999) and Ara h 4 (Kleber-Janke et al., 1999) indicating that these proteins were in fact variants of the same gene. Ara h 4 was later renamed Ara h 3.02 (Radauer et al., 2014). Ara h 3 is post-translationally cleaved into a 43 kDa acidic and a 28 kDa basic subunit that are covalently linked by a disulfide bond (Schmidt et al., 2009). In summary, several fragments of Ara h 3 (14, 25, 42 and 45 kDa) can be observed, even under extraction conditions that inhibit proteases (Koppelman et al., 2003). This illustrates that Ara h 3 is proteolytically processed in peanuts. An additional isoform, iso-Ara h 3, only shares 70–85% sequence identity with the other reported Ara h 3 isoforms (Boldt et al., 2005). In fact, five different genes were described to encode isoforms of Ara h 3 (Yan et al., 2005).

### 3.2 2S albumins: Ara h 2, Ara h 6, Ara h 7

Ara h 2, a 2S albumin, can be purified as a doublet as described by Burks et al. (Burks et al., 1992) and de Jong et al. (de Jong et al., 1998), both bands having the same N-terminal sequence. An almost complete cDNA sequence of Ara h 2.01 was published in 1997 by Stanley et al. (Stanley et al., 1997), and in 2003 complete cDNA sequences for both Ara h 2.01 and Ara h 2.02 were made available by Chatel and colleagues (Chatel et al., 2003). The isoform Ara h 2.02 is characterized by a 12 amino acid residue insertion at position 75 in comparison to the isoform Ara h 2.01. The deduced amino acid sequence of a full length intron-free genomic clone of Ara h 1.01 comprises 207 residues and includes a signal peptide of 21 residues (Viquez et al., 2001). The two isoforms of Ara h 2 are expressed from different genes. Furthermore, Ara h 2 undergoes proteolytic processing by peanut proteases resulting in the removal of the dipeptide RY at the C-terminus (Radosavljevic et al., 2010). Consequently, Ara h 2 is a mix of two isoforms as well as slightly truncated forms of both isoforms.

The first cloning of a cDNA coding for an Ara h 6 isoform, another 2S albumin, was reported in 1999 by Kleber-Janke and colleagues (Kleber-Janke et al., 1999). This cDNA

expression product was identified by phage surface display technology. Ara h 6 contains 10 cysteine residues. Suhr et al. identified three different isoforms of natural (n) Ara h 6 isolated from crude peanut extract by N-terminal amino acid sequencing (Suhr et al., 2004). The presence of a signal peptide was predicted by comparing the N-terminus of the natural protein with the deduced amino acid sequence from the cDNA clone AF092846. Another nAra h 6 isoform from crude peanut extract was reported by Koppelman et al. (Koppelman et al., 2005). Bernard and colleagues published two additional nAra h 6 isoforms (Bernard et al., 2007). The native form of this Ara h 6 isoform occurs together with its naturally processed form in peanut. The processing results in the loss of the dipeptide IR at positions 46 and 47 of the mature protein and the formation of two peptide chains of 5.44 and 9.14 kDa linked together by disulfide bonds. *Arachis hypogaea*, being an allotetraploid with an AABB genome constitution (Bertioli et al., 2016), carries three copies of the *arah6* gene, one of them located in the A genome and the other two in the B genome (Ramos et al., 2006).

An Ara h 7 cDNA sequence was first cloned by using the pJuFo phage display system (Kleber-Janke et al., 1999) and deposited in the database with the accession number AF091737. Ara h 7 is related to the other two 2S albumin allergens, Ara h 2 and Ara h 6, but the isoform Ara h 7.0101 only possessed 6 cysteine residues. Ara h 7 was later recloned and its sequence (EU046325) was accepted as the 17.7 kDa isoallergen Ara h 7.0201 (Schmidt et al., 2010). Ara h 7.0201 possessed the conserved cysteine skeleton of 8 cysteine residues. Ara h 7.0201 could also be identified as a natural protein present in peanuts, while the previously annotated Ara h 1.0101 could not be found. A third isoallergen, Ara h 7.0301 (AY722691) was identified by expressed sequence tag analysis (Yan et al., 2005).

### 3.3 Profilin: Ara h 5

The first cDNA coding for the peanut profilin Ara h 5 was obtained from a pJuFo phage surface display library that had been derived from a  $\lambda$ -ZAPII library (Kleber-Janke et al., 1999). The cDNA's coding region comprised 396 nucleotides, predicting a protein of 131 amino acid residues with a calculated mass of 14 kDa. The sequence was deposited in the GenBank database with the accession number AF059616. This approach was expanded in a follow-up study where 25 clones carrying Ara h 5 cDNAs ranging in length from 450 to 750 base pairs were isolated (Kleber-Janke et al., 2001). All 25 clones carried cDNA inserts coding exclusively for one protein whose amino acid sequence is available under the accession number AAD55587. Ara h 5 was recloned in 2010 in Japan resulting in the protein sequence GU354312 (Cabanos et al., 2010a) that was 94.7% identical to the one previously published by Kleber-Janke et al. (Kleber-Janke et al., 1999).

### 3.4 PR-10: Ara h 8

A cDNA encoding the Bet v 1-homologous Ara h 8 was amplified by PCR using degenerate primers designed on the basis of the sequence of a soybean PR-10 protein, Gly m 4 (Mittag et al., 2004). The full-length cDNA sequence (AY328088) harbored a 471 base pair open reading frame coding for a protein of 157 amino acid residues with a predicted molecular weight of 16.9 kDa. The protein sequence resulting from this sequence was assigned the isoallergen designation Ara h 8.0101. The characterization by micro-sequencing of a natural Ara h 8 protein isolated from peanuts revealed differences to the deduced amino acid

sequence AY328088 (Riecken et al., 2008). The cDNA of this new Ara h 8 isoallergen was cloned, its sequence deposited in the database under the accession number EU046325, and the corresponding allergen designated as Ara h 8.0201. There is a similarity of only 51.3% between the two isoallergens. Analysis of genomic DNA obtained with Ara h 8.0101-specific primers revealed the presence of one intron (Riecken et al., 2008).

### 3.5 Non-specific lipid transfer proteins type 1 and type 2: Ara h 9, Ara h 16, Ara h 17

Full-length cDNAs of two Ara h 9 isoforms, non-specific lipid transfer proteins, were cloned using a combination of molecular biology and bioinformatics tools (Krause et al., 2009). A signal peptide of 24 amino acid residues was predicted for both isoforms which was confirmed by N-terminal sequencing of natural peanut nsLTP. The two nsLTP isoforms shared 90% sequence identity and were named Ara h 9.0101 (9.135 kDa) and Ara h 9.0201 (9.054 kDa). The sequences were made available with the GenBank accession numbers EU159429 and EU161278.

Two additional non-specific lipid transfer proteins were accepted by the WHO/IUIS allergen nomenclature sub-committee (<http://www.allergen.org>) but the respective papers have not yet been published. Ara h 16.0101 is a type 2 nsLTP with a calculated molecular weight of 7 kDa and Ara h 17.0101 is a type 1 nsLTP with a calculated molecular weight of 9.4 kDa.

### 3.6 Oleosins: Ara h 10, Ara h 11, Ara h 14, Ara h 15

Oleosins are the major oil body stabilizing proteins. At least eight peanut-derived oleosins have been identified on the DNA level and by proteomic approaches (Jolivet et al., 2013; White et al., 2013). Schwager and coworkers have identified eight allergenic peanut oleosins by using N-terminal sequencing and peptide mass fingerprinting (Schwager et al., 2015). These eight oleosins were classified into four allergen groups, i.e. Ara h 10 comprising the isoforms Ara h 10.0101 (Q647G5, predicted molecular weight 17.6 kDa) and Ara h 10.0102 (Q647G4, 15.4 kDa); Ara h 11 comprising the two isoforms Ara h 11.0101 (Q45W87, 15.4 kDa) and Ara h 11.0102 (Q45W86, 15.4 kDa); Ara h 14 comprising the three isoforms Ara h 14.0101 (Q9AXI1, 16.5 kDa), Ara h 14.0102 (Q9AXI0, 16.5 kDa), and Ara h 14.0103 (Q6JIJ8, 16.5 kDa); and Ara h 15 with the isoform Ara h 15.0101 (Q647G3, 15.4 kDa).

### 3.7 Defensins: Ara h 12, Ara h 13

N-terminal sequencing of IgE reactive proteins extracted by chloroform/methanol from roasted peanuts followed by a homology search in the expressed sequence tag database led to the identification of defensins as peanut allergens (Petersen et al., 2015). Two groups of peanut defensins with sequence identities of 43% to 45% were found. Ara h 12 is represented by one isoform (EY396089) with a molecular mass of 5.2 kDa. Two Ara h 13 isoforms, with determined molecular masses of around 5.4 kDa, were identified. Ara h 13.0101 (EY396019) and Ara h 13.0102 (EE124955) differed by only three amino acid residues.

## 4 Expression of recombinant peanut allergens

Peanut allergens have been expressed in various pro- and eukaryotic expression systems. An overview on these expression systems, the plasmids used and the yields is given in Table 2.

Hurlburt and colleagues back-translated the Ara h 1 protein sequence using optimized codons for the expression in *E. coli* (Hurlburt et al., 2014). The authors established a reproducible protocol to produce large quantities of pure rAra h 1. Mature Ara h 1 (residues 85–626) and the Ara h 1 core domain (residues 170–586) were expressed in BL21(DE3) cells using the pET-9a vector. A little more than half of the expressed protein appeared in the soluble fraction. Compared to natural Ara h 1, both recombinant Ara h 1 forms showed lower IgE binding by the majority of patients' sera used in the study.

The presence of codons in an allergen's cDNA that are rarely used in *E. coli* leads to decreased expression levels, frame shifts and mistranslations. The BL21(DE3) *E. coli* strain is a convenient and effective host for heterologous protein expression. However, when the cDNAs coding for Ara h 1, Ara h 2 and Ara h 6 inserted in pET-16b were expressed in BL21(DE3) cells, production was very inefficient (Kleber-Janke and Becker, 2000). In contrast, the peanut profilin Ara h 5 was successfully expressed. Ara h 1 has a content of 5.4% of the arginine codons AGG and AGA that are least used in *E. coli*, Ara h 2 has 90.6%, and Ara h 6 has 8.1%. The AGG/AGA content of Ara h 5 is only 0.8%. Transfer RNAs that recognize these codons are extremely rare in *E. coli*. A high level of expression of these allergens could be achieved by using *E. coli* BL21(DE3)-Codon Plus RIL cells that carry extra copies of the argU tRNA gene (Kleber-Janke and Becker, 2000). The expression yield of Ara h 5 which was totally present in the soluble fraction was 30 mg/L culture. The yield of Ara h 2.01 from the soluble fraction was 4.5 mg/L culture and from inclusion bodies 24 mg/L.

Lehmann and colleagues reported a method for large scale production of properly folded Ara h 2 (Lehmann et al., 2003). The full-length coding sequence of Ara h 2.01 was inserted into the pET-32a expression vector fused at the N-terminus with the sequence encoding the 109 residues of the thioredoxin tag. *E. coli* Origami (DE3) cells were cotransformed with the expression vector and a plasmid carrying the argU tRNA gene. The thioredoxin fusion tag was used to enhance the solubility of rAra h 2 and to catalyze the formation of disulfide bonds in the oxidizing cytoplasm of this modified *E. coli* strain. rAra h 2 was expressed at 19 mg/L culture and was shown to be identical to natural Ara h 2 as judged by immunoblotting, analytical high-performance liquid chromatography (HPLC) and circular dichroism spectra.

A codon-optimized gene of Ara h 2.01 was inserted in the pMALX\_E plasmid resulting in the expression of Ara h 2 fused to a mutated version of the maltose binding protein (MBP) (Mueller et al., 2011). *E. coli* Origami B cells were serially transformed with a pACYCDuet-1 plasmid encoding thioredoxin followed by pMALX\_E Ara h 2 plasmids. The purified MBP-Ara h 2 protein was then used for crystallization trials.

Lew et al. expressed a codon-optimized Ara h 2.02 gene inserted into a pET-28a(+) as an N-terminally hexahistidine-tagged protein in *E. coli* BL21(DE3) cells (Lew and Lim, 2016).

The insoluble recombinant protein was isolated under denaturing conditions by Ni-affinity chromatography and refolded by dialysis in decreasing urea concentrations. The rAra h 2.02 that was produced at a yield of 74 mg/L culture showed a significantly decreased reactivity with specific IgE when compared to natural Ara h 2.

*Lactococcus lactis* is a gram-positive lactic acid bacterium with food grade status, free of endotoxins, and the ability to secrete heterologous proteins with few other proteins. The Ara h 2.02 gene codon optimized for expression in *L. lactis* was inserted into the expression vector pAMJ399 as a fusion to a signal peptide to enable its secretion into the culture medium (Glenting et al., 2007). Correctly processed full-length Ara h 2.02 was isolated at 40 mg/L culture.

Heterologous proteins can be produced in chloroplasts of the unicellular eukaryotic green alga *Chlamydomonas reinhardtii* (Mayfield et al., 2003). *C. reinhardtii* can be rapidly transformed into stable transgenic strains and grown in large quantities in minimal media in photobioreactors. Codon optimization was done for Ara h 1 and Ara h 2.02 for the codon usage of *C. reinhardtii* chloroplasts, cloned into pJAG15 and the transformed algae were grown in large-scale cultures (Gregory et al., 2016). Although the core domain of Ara h 1 and the full-length Ara h 2.02 were produced in algal chloroplasts, the recombinant proteins displayed a reduced binding to IgE from peanut allergic patients as compared to the native allergens. This could indicate a possible lack of post-translational modifications.

*E. coli* Rosetta2(DE3) is a BL21 derivative designed to alleviate codon bias when expressing heterologous proteins in *E. coli*. The gene encoding Ara h 5 was cloned into the pET-21d plasmid and expressed in Rosetta2(DE3) cells (Cabanos et al., 2010a). The total yield of the purified protein was 29 mg/L culture. When tested in a microarray together with birch and timothy profilins, rAra h 5 displayed good cross-reactivity with the two pollen profilins.

One of the advantages of *Pichia pastoris* over *E. coli* is its ability to produce more properly formed disulfide bonds. Ara h 6 contains 10 cysteine residues that form 5 disulfide bonds required for correct folding and allergenic activity. The Ara h 6 gene, codon-optimized for yeast, was cloned into the pPink-HC expression vector and transformed into *PichiaPink* cells (Zhuang et al., 2012). Compared to natural Ara h 6, rAra h 6 produced in *Pichia* had intact effector functions while Ara h 6 produced in *E. coli* BL21(DE3)-Codon Plus RIL cells via the pET-32 Ek/LIC vector had significantly reduced functions.

Due to the difficulties of obtaining soluble oleosins in *E. coli*, the peanut oleosins Ara h 10, Ara h 11, and Ara h 14 were expressed in soluble form in the insect cell-baculovirus system (Cabanos et al., 2011a). The coding genes of the peanut oleosins were inserted into the pENTR4 vector which was then recombined with BaculoDirect Linear DNA. Recombinant baculovirus constructs were then transfected into *Spodoptera frugiperda* Sf9 cells. All three oleosins were expressed in soluble form in the insect cells. The final protein yield was 0.9 mg/L of culture for Ara h 10, 0.8 mg/L for Ara h 11, and 1.3 mg/L of culture for Ara h 14.



## 5 Purification of natural peanut allergens

Natural allergens, too often disregarded, are the authentic counterpart of recombinant allergens. They are absolutely required for the validation of the quality of newly produced recombinant allergens. However, purification of natural allergens from peanut can be quite challenging. In addition, food allergens undergo modifications during food processing which are not present in recombinant allergens. Peanuts are consumed after thermal processing, such as boiling, frying or roasting, and as a consequence, the physicochemical properties of their allergens change impacting on their allergenicity and IgE-binding capacity (Guillon et al., 2016; Zhang et al., 2016; Cabanillas et al., 2012).

Peanut proteins make up 22–30% of the total protein in peanut seeds (Sebei et al., 2013). Sixteen proteins, belonging to 7 protein families, are at present classified as allergens (<http://www.allergen.org>). Many peanut allergens are seed storage proteins that, being closely related, are challenging to purify. Other allergens are much less abundant in seeds and are hardly obtained in decent amounts to work with. Several studies have contributed to the identification and characterization of peanut allergens in crude extracts, and many provided us with a detailed purification method for obtaining pure allergens, sometimes in high yields. Here, we describe some of the purification methods made available for most of the allergens, or their identification in crude peanut extracts. Table 3 gives an overview on purification procedures.

### 5.1 Cupins: Ara h 1, Ara h 3

The peanut seed storage allergens, Ara h 1 (Burks et al., 1991) and Ara h 2 (Burks et al., 1992) make up 12–16% and 5.9–9.2% of the total peanut protein content, respectively (Koppelman et al., 2001). Ara h 1 is a glycoprotein with a molecular mass of 63.5 kDa, forming trimers of 180 kDa (Chruszcz et al., 2011). It can be purified from crude peanut extract by affinity chromatography to concanavalin A (ConA) which binds the mannose residues present on the glycan chain (Eiwegger et al., 2006). A higher purity level of Ara h 1 can be achieved by size exclusion chromatography following affinity chromatography (Marsh et al., 2008).

Ara h 3, with a molecular mass of 60 kDa for the monomer, occurs in peanuts as hexamer of 360 kDa. Koppelman et al. developed a strategy for the purification of natural Ara h 3 from crude peanut extract and described a post-translational processing of Ara h 3 that affects its IgE-binding properties (Koppelman et al., 2003).

### 5.2 2S albumins: Ara h 2, Ara h 6, Ara h 7

Ara h 2, described as the most potent peanut allergen (Koppelman et al., 2004) and the best predictor of peanut allergy in adults (Klemans et al., 2013), was purified by Burks and coworkers by ion exchange chromatography (Burks et al., 1992). There are two isoforms of Ara h 2, Ara h 2.0101 and Ara h 2.0201, and their molecular masses are 16.7 and 18.0 kDa, respectively. Ara h 2.0201 differs from the other isoform by an insertion of 12 amino acids residues (Hales et al., 2004). In 2008, Marsh and colleagues obtained pure Ara h 2 from peanut seeds by fractionating a peanut protein extract with ammonium sulfate followed by

size exclusion chromatography, anion-exchange and preparative C18 RP-HPLC (Marsh et al., 2008). A simplified procedure was proposed by Masuyama and colleagues (Masuyama et al., 2014) which enabled the purification of Ara h 2 without the use of chromatography.

Ara h 6 is of high clinical importance as, together with Ara h 2, it is the best predictor of severe peanut allergy (Kukkonen et al., 2015). Ara h 6 was isolated from peanut extracts by size exclusion chromatography, in which Ara h 6 was co-eluted with Ara h 2, followed by an anion-exchange chromatography (Suhr et al., 2004). In 2008, Marsh et al. proposed a different protocol for the purification of both Ara h 2 and Ara h 6 from peanut seeds that allows a clear separation of the two allergens, described above in more detail for Ara h 2 (Marsh et al., 2008).

Ara h 7, like Ara h 2 and Ara h 6, is a 2S albumin with a molecular mass of 17.3 kDa. Due to their cross-reactivity and their similar physiochemical properties, the identification of this natural allergen was challenging. Schmidt et al. were able to identify only Ara h 7.0201 in peanut extract from a pool of size exclusion chromatography-enriched 20 kDa proteins separated by 2D gel electrophoresis (Schmidt et al., 2010).

### 5.3 PR-10: Ara h 8

Ara h 8, the Bet v 1 homologue from peanut, was identified in 2004 by Mittag and coworkers (Mittag et al., 2004). However, only 4 years later, a purification strategy for obtaining pure Ara h 8 from peanut extract was published (Riecken et al., 2008). Ara h 8, with a molecular mass of 17 kDa is not abundant in peanut seeds. Thus, the authors developed a protein extraction method using an acidic buffer which resulted in an Ara h 8-enriched extract. A different purification system for Ara h 8 was proposed by Petersen et al., based on a lipophilic extraction of proteins from peanuts, to achieve a yield of 20 and 8 µg from 1 g of unroasted and roasted peanut flour, respectively (Petersen et al., 2014).

### 5.4 Non-specific lipid transfer proteins type 1 and type 2: Ara h 9, Ara h 16, Ara h 17

The peanut nsLTP Ara h 9, an allergen important for the Mediterranean population, was purified by Lauer et al. by a two-step purification procedure (Lauer et al., 2009). It is a small 9.1 kDa protein with a basic pI of 9.3. The authors obtained 1.3 mg of pure Ara h 9 starting from 110 g of peanuts. Two additional peanut nsLTPs are listed in the WHO/IUIS allergen nomenclature database (<http://www.allergen.org>), named Ara h 16 and Ara h 17, but so far no data on their purification strategies are available.

### 5.5 Oleosins: Ara h 10, Ara h 11, Ara h 14, Ara h 15

Oleosins are very abundant in peanut seeds. In 2015, Schwager et al. published a method for the simultaneous purification of all known peanut oleosins (Schwager et al., 2015). The purification method is based on the isolation of oil bodies from peanut, which were delipidated and then peanut oil body proteins were separated by preparative electrophoresis. In this study, the authors identified Ara h 14 and Ara h 15, which were then officially accepted as allergens.

## 5.6 Defensins: Ara h 12, Ara h 13

Recently, Ara h 12 and Ara h 13, two defensins with antifungal activity, were identified as novel peanut allergens and purified after a lipophilic extraction (Petersen et al., 2015).

## 6 Effects of thermal processing on peanut allergens

Peanuts undergo thermal processing before consumption. They are eaten boiled, fried or roasted according to various culinary traditions. These preparation methods seem to have an impact on the prevalence of peanut allergy. In fact, a lower incidence of peanut allergy is reported in countries where peanuts are consumed after boiling (Beyer et al., 2001). Thermal processing has been shown to affect peanut proteins in different manners. Boiling of peanuts, a cooking method widely used in China, decreases the IgE binding capacity of Ara h 1, Ara h 2, and Ara h 3 compared to roasting (Beyer et al., 2001). This is probably due to a loss of allergens into the boiling water (Mondoulet et al., 2005). Moreover, the IgE-binding of sera from patients tolerant to boiled peanuts was reduced and mostly limited to the peanut 2S albumins Ara h 2, 6 and 7 (Turner et al., 2014).

The process of roasting was shown to increase peanut allergenicity and the IgE-binding capacity of peanut allergens (Beyer et al., 2001; Mondoulet et al., 2005). The Maillard reaction which occurs during dry-roasting between the allergens' amino groups and reducing sugars present in peanuts contributes to this effect.

Consequently, the allergens Ara h 1 and Ara h 2 undergo chemical modifications which increase their IgE-binding capacity, produce more stable structures and confer resistance to heat and digestion (Maleki et al., 2000). The presence of advanced glycation end products in roasted peanuts might explain the higher levels of IgE-binding compared to boiled or fried peanuts (Chung and Champagne, 2001). Furthermore, roasting of peanuts was necessary to induce sensitization to Ara h 6 in mice as unroasted Ara h 6 could not (Guillon et al., 2016). The origin of this difference might be found in the formation of a stable protein complex between Ara h 6 and Ara h 1 in roasted peanut extracts. This effect on increased IgE-binding due to roasting was also described for the peanut Bet v 1 homologue Ara h 8 (Petersen et al., 2014). Ara h 8 turned out to be more stable to heat and enzymatic digestion, possibly as a consequence of the association with lipophilic ligands stabilized by roasting.

Allergenicity of peanut proteins is also affected by structural changes induced by thermal processing. Frying of peanuts, but not boiling or roasting, altered the secondary structure of Ara h 2 dramatically by decreasing the molecule's content of  $\alpha$ -helices and increasing its  $\beta$ -sheets,  $\beta$ -turn and random coil, thus altering Ara h 2 epitopes and reducing its allergenicity (Zhang et al., 2016). In addition, boiling of Ara h 1 induced a partial loss of secondary structure of the molecule which then assembled into branched complexes with a reduced IgE-binding capacity due to decreased epitope availability (Blanc et al., 2011). Although boiled peanuts had a reduced allergenicity, they could not be regarded as hypoallergenic peanuts (Comstock et al., 2016). Although many studies investigated the effect of thermal processing on the IgE-binding properties of peanuts, the contribution of food processing to the immunological characteristics of peanut allergens and peanut matrix components needs to be further elucidated.

## 7 Post-translational modifications

Post-translational modifications (PTMs) refer to covalent modifications of proteins once they have been fully translated. PTMs such as phosphorylation, acetylation or glycosylation control many biological processes. As many as 300 PTMs of proteins are known to occur physiologically (Witze et al., 2007). The study of PTMs and their functions in plants is an emerging field (Hashiguchi and Komatsu, 2017). Glycosylation is the major PTM of peanut allergens that has been studied to date. Ara h 1 contains one glycosylation site that bears mainly xylosylated *N*-glycans of the composition  $\text{Man}_{3(-6)}\text{XylGlcNAc}_2$  (Kolarich and Altmann, 2000). While the predicted glycosylation of Ara h 2 was not found, site-specific hydroxylations were identified of 2/8 and 3/11 proline residues of Ara h 2.01 and Ara h 2.02, respectively (Li et al., 2010). In contrast to Ara h 2, no consensus sequence for *N*-glycosylation could be identified in the sequence of Ara h 6 (Suhr et al., 2004). Ara h 6 was also assumed not to be a glycoprotein based on data obtained by Zhuang et al (Zhuang et al., 2012). Although putative glycosylation sites are present in Ara h 12 and Ara h 13, mass spectrometry proved that these peanut defensins were not glycosylated (Petersen et al., 2015).

## 8 Available structures of peanut allergens

X-ray crystallography structures are available for five peanut allergens (Table 4). While structures were obtained for four peanut allergens, Ara h 1, 2, 5, and 8, from recombinant proteins, the structure of the hexameric Ara h 3 was obtained from a natural protein. Fig. 1 shows the ribbon representations of the architecture of these allergens.

While the full-length recombinant protein did not exhibit a fully native structure and was partially unfolded, the crystal structure of the Ara h 1 core (residues 170–586) could be determined at resolutions of 2.71 and 2.35 Å (Chruszcz et al., 2011). The core region of Ara h 1, which revealed the typical bicupin fold, formed homotrimers and existed as higher molecular weight assemblies in solution. This was in agreement with the observations made by van Boxtel and colleagues (van Boxtel et al., 2006). A second study on the crystallization of Ara h 1 was performed by Cabanos and colleagues (Cabanos et al., 2011b). Comparable to the Chruszcz study, a full-length Ara h 1 complete with the N-terminal extension (residues 1–86) and the C-terminal flexible region (residues 504–536) did not yield crystals with a good diffraction quality. However, the crystal structure of an Ara h 1 core region (residues 145–562, Cabanos et al., 2010b) was determined at a final resolution of 2.43 Å. Ara h 1 was found to exist in trimeric form and its structure and topology were very similar to other known structures of 7S globulins.

Ara h 2 was crystallized as a fusion protein that used an engineered maltose-binding protein as a carrier to improve the likelihood of crystallization (Mueller et al., 2011). Hence, in the crystal structure deposited in the PDB database the Ara h 2 residues are numbered 1028–1148 (PDB 3OB4). The crystal structure determined at a resolution of 2.7 Å revealed a bundle of five alpha-helices held together by four disulfide bonds typical for the small molecular weight members of the prolamin superfamily.

Diffraction quality crystals of Ara h 3 were obtained by purifying one isoform from dry peanut kernels (Jin et al., 2007). Ara h 3 is a bicupin hexamer, the trimer to hexamer transition is only possible after the cleavage of the peptide bond between the acidic and the basic subunit. Due to the purification of the natural protein, the crystallization of a homohexameric Ara h 3 at a resolution of 1.73 Å was achieved (Jin et al., 2009). Simply expressing the coding sequence of Ara h 3 in an expression vector would not have yielded a recombinant protein in its native hexameric state.

The structure of a peanut profilin was determined at a resolution of 1.10 Å for recombinant Ara h 5 produced in *E. coli* (Wang et al., 2013). The structure displayed the typical profilin-like fold consisting of a seven-stranded antiparallel β-sheet with two α-helices on one side and one α-helix on the other side. Although the *H. brasiliensis* latex profilin Hev b 8 had the highest percentage of sequence identity with Ara h 5, the structural alignment with the birch pollen profilin was much better. This indicates that a model-based prediction of IgE epitopes needs to be approached with caution.

Recombinant Ara h 8 was expressed in *E. coli* and the structure of the purified protein was determined at a resolution of 1.60 Å (Hurlburt et al., 2013). Its overall fold consisted of three α-helices that face a seven-stranded anti-parallel β-sheet illustrating the high similarity to the birch pollen allergen Bet v 1 architecture despite a low sequence identity of 48%. Like Bet v 1, Ara h 8 contained a large hydrophobic cavity.

## 9 Use of peanut allergens in diagnosis

The double-blind placebo-controlled food challenge (DBPCFC) is and remains the gold standard of food allergy diagnosis (Sampson et al., 2012). However, when testing for the presence of peanut allergy, the DBPCFC bears an inherent risk of inducing life-threatening reactions (van Erp et al., 2013). In many cases, the presence of measurable levels of specific IgE to a food will help establish a diagnosis of food allergy. Hence, the availability of purified allergens allows the possibility of multiple testing and determination of specific IgE (sIgE) levels against allergens in one single measurement (Villalta et al., 2018). For peanut allergic patients, who often manifest severe reactions to minute amounts of allergens, the so-called component-resolved diagnosis (CRD) is a valuable diagnostic tool. Instead of exposing patients to crude allergen extracts, CRD utilizes purified natural or recombinant allergens to identify the specific molecule involved in sensitization or allergy (Borres et al., 2016).

The allergens available for CRD on the ImmunoCAP® ISAC are Ara h 1, Ara h 2, Ara h 3, Ara h 6, Ara h 8, and Ara h 9. The major peanut allergen Ara h 2 is associated with the severity of symptoms of peanut allergy (Kukkonen et al., 2015). It was shown that sIgE to Ara h 2 could be used as a good predictor of suspected peanut allergy, for both children and adults, among allergic populations of several geographic regions (Klemans et al., 2015). A similar diagnostic value of sIgE was shown for the peanut allergen Ara h 6 (Klemans et al., 2014), probably due to its homology to the 2S albumin Ara h 2 (Koppelman et al., 2005).

In contrast, studies performed with the peanut allergens Ara h 1 and Ara h 3 showed that the predictive diagnostic value of their sIgE was quite low and depended on the geographical area of the origin of the study populations (Ballmer-Weber et al., 2015; Klemans et al., 2015). Some of the peanut allergens, such as Ara h 5 and Ara h 7, are neither available nor have they been investigated in clinical studies which may represent a limit of the CRD. The same applies to the peanut oleosins, which were only recently associated with severe peanut allergic reactions (Schwager et al., 2017). It is also important to consider whether a patient might be at risk of developing symptoms when eating peanuts because of cross-reactivity of certain allergens with other homologous allergens. This is the case for Ara h 8 which cross-reacts with IgE induced by the birch pollen allergen Bet v 1 (Asarnoj et al., 2010; Mittag et al., 2004) and for Ara h 9 which cross-reacts with other nsLTPs (Krause et al., 2009).

## 10 Vaccine candidates

At present, no routine therapeutic approaches are available for the treatment of peanut allergy. Strict avoidance of the culprit food is the only available option. A possible therapeutic line of action might be the modulation of the immune response to peanut allergens and the induction of oral tolerance. However, one of the major problems during attempts of treating food allergies is the occurrence of adverse reactions (Jones et al., 2014). Recombinant hypoallergenic allergen variants offer an alternative approach to avoid such IgE-mediated adverse reactions (Tschepppe and Breiteneder, 2017; Satitsuksanoa et al., 2018).

In 2001, Bannon and colleagues produced hypoallergenic variants of Ara h 1, Ara h 2 and Ara h 3 in *E. coli* showing a reduced IgE-binding capacity of the newly produced molecules by immunoblotting with peanut allergic patients' sera (Bannon et al., 2001). Due to the clinical relevance of Ara h 2, more efforts were made to produce a recombinant hypoallergenic variant of Ara h 2. All linear IgE-binding epitopes of Ara h 2 were modified to reduce its IgE-binding capacity (King et al., 2005). The modified Ara h 2 displayed a reduced capacity to trigger the release of  $\beta$ -hexosaminidase from an RBL-2H3 cell line passively sensitized with IgE from peanut allergic individuals.

A hypoallergenic derivative of Ara h 3 was produced by introducing point mutations into four critical IgE-binding sites while the ability to stimulate T-cell proliferation was retained (Rabjohn et al., 2002). In a mouse model of peanut allergy, three modified peanut allergens, Ara h 1–3, were subcutaneously co-administered with heat-killed *Listeria monocytogenes* bacteria (Li et al., 2003). This treatment markedly decreased peanut-specific IgE and histamine levels in the plasma and offered protection from anaphylaxis. The exact mechanism conveying protection was not clear but the effect was accompanied by a switch from a Th2- to a Th1-biased response (Table 5).

## 11 Clinical trials

In 2013, a mix of recombinant hypoallergenic variants of the peanut allergens Ara h 1, 2 and 3, called EMP-123, entered phase I of an open-label clinical trial (Wood et al., 2013). The three recombinant allergens were encapsulated in heat/phenol-inactivated *E. coli* cells used

as adjuvant. The candidate vaccine EMP-123 was administered rectally to 10 peanut allergic adults in weekly doses over 10 weeks, followed by 3-weekly doses. Of the 10 patients enrolled, 5 experienced adverse reactions and dropped out of the study. The reason for the observed adverse reactions might have been due to an incomplete removal of the IgE-binding epitopes from the allergens.

A first multicenter, randomized, double-blind placebo-controlled study of peanut sublingual immunotherapy (SLIT) was published by Fleischer and colleagues in 2013 (Fleischer et al., 2013). Forty subjects were enrolled in this study, with ages ranging from 12 to 37 years. After 44 weeks of SLIT, 14 of 20 subjects receiving peanut SLIT were responders compared to 3 of 20 subjects receiving placebo. In the responders, the median successfully consumed dose increased from 3.5 to 496 mg. Peanut SLIT induced a modest level of desensitization in a majority of the subjects compared to the placebo group.

The LEAP (Learning Early About Peanut Allergy) study was a clinical trial completed in order to evaluate strategies whether peanut consumption or avoidance would be more effective in preventing the development of peanut allergy in children at high risk (Du Toit et al., 2015). Six hundred and forty atopic children with eczema or egg allergy, not younger than 4 months and not older than 11 months, were enrolled for this study and randomly assigned to avoidance or peanut consumption. In the consumption group only 1.9% of the children developed peanut allergy at the age of 60 months, whereas 13.7% developed peanut allergy in the avoidance group. According to the LEAP study, the early introduction of peanuts in the diet of children at high risk is beneficial for reducing the frequency of the development of peanut allergy.

In a single center clinical trial, 37 children who were aged 9–36 months and who had a reaction during the entry open food challenge either received low-dose (target maintenance dose: 300 mg/d peanut protein) or high-dose (3000 mg/d) early intervention oral immunotherapy (E-OIT). (Vickery et al., 2017). Overall, 78% of the subjects receiving E-OIT demonstrated sustained unresponsiveness to peanut four weeks after stopping E-OIT after a median of 29 months of treatment enabling them to reintroduce peanut-containing foods into the diet *ad libitum*. Interestingly, 300 mg/d was as effective as 3000 mg/d at regulating the allergic immune response. In accordance with the LEAP study, this E-OIT trial suggested that allergic responses may be more easily corrected in young children.

A Viaskin Peanut patch (DBC Technologies SA) comprising of an epicutaneous delivery system containing a dry deposit of a formulation of peanut extract was applied in a multicenter, randomized DBPCFC phase II study (Jones et al., 2017). Of the 74 peanut allergic participants, 24 were assigned to the group treated with Viaskin Peanut 100 µg (VP100), 25 were treated with Viaskin Peanut 250 µg (VP250), and 25 subjects were assigned to the placebo group. Treatment success after 52 weeks was defined as passing a 5044 mg peanut protein OFC or achieving a 10-fold greater increase in peanut protein consumption from baseline. Forty-six percent of the VP100 participants, 48% of the VP250 participants, but only 12% of the placebo group achieved the treatment success.

A phase IIb DBPC dose-ranging study of a peanut patch was performed in 221 peanut allergic patients, aged 6 to 55 years, from 22 centers (Sampson et al., 2017). Patients received epicutaneous peanut patches containing 50 µg (n = 53), 100 µg (n = 56), or 250 µg (n = 56) peanut protein or a placebo patch (n = 56) for 12 months. Following the therapy, changes in the eliciting dose were established for each patient by DBPCFC. The 250 µg peanut patch resulted in a significant treatment response versus the placebo patch. The largest effect was seen in children with approximately 50% achieving the primary endpoint at 12 months defined by either a 10-times increase in the challenge threshold or an increase of the symptom-eliciting dose to 100 mg peanut protein or more.

Recently, the first phase II randomized, double-blind, placebo-controlled study was carried out in eight US centers to assess the safety and efficacy of AR101, a novel oral biologic drug (Bird et al., 2018). AR101 consisted of encapsulated defatted lightly roasted peanut flour, well characterized in terms of peanut allergen composition and potency. Fifty-five peanut sensitized individuals were enrolled in the study, between 4 and 26 years old, with symptoms triggered by less than 143 mg/day as assessed by DBPCFC. Patients were exposed daily to AR101 or placebo with an increased dosage from 0.5 mg to 300 mg/day. AR101 significantly improved patients' symptoms by reducing the severity of symptoms following DBPCFC. Patients tolerated 18-fold higher amounts of peanut proteins after the treatment (Table 6).

## 12 Future directions

Peanut allergens will continue to generate research interests. The recombinant production of peanut allergens will encompass the more recently described peanut allergen such as the oleosins, defensins, and nsLTGs. At present, there are no structures available. It will prove to be quite challenging to complete the full panel of structures. Likewise, the allergenic characteristics and immunological properties of the peanut oleosins, defensins, and nsLTGs will have to be determined in broader studies. It will be advisable to validate the qualities of the recombinant peanut allergens using their purified natural counterparts. It is unclear at present, how much post-translational modifications contribute to the allergenic potency and whether there are such modifications that have been overlooked. The effect of thermal processing on the more recently described peanut allergens is yet another area that will have to be investigated. Last but not least, the contribution of the matrix, i.e. the peanut lipids, to the sensitization process is not well understood. Studies of the effects of peanut matrix components in association with the allergens might shed more light on the mechanisms that ultimately result in the production of allergen specific IgE.

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## Abbreviations

**CRD** component resolved diagnosis



<b>DBPCFC</b>	double-blind placebo-controlled food challenge
<b>FPLC</b>	fast protein liquid chromatography
<b>HIC</b>	hydrophobic interaction chromatography
<b>HPLC</b>	high performance liquid chromatography
<b>IEC</b>	ion exchange chromatography
<b>MBP</b>	maltose-binding protein
<b>nsLTP</b>	non-specific lipid transfer protein
<b>OIT</b>	oral immunotherapy
<b>PBMC</b>	peripheral blood mononuclear cells
<b>PTM</b>	post-translational modification
<b>SEC</b>	size exclusion chromatography
<b>SLIT</b>	sublingual immunotherapy
<b>SPT</b>	skin prick test

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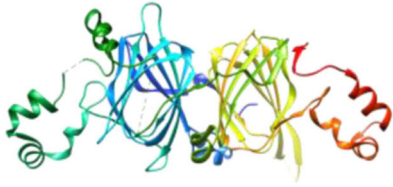
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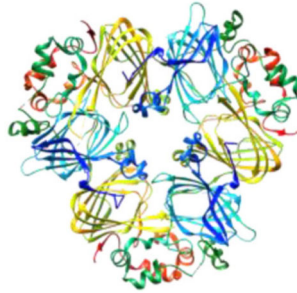
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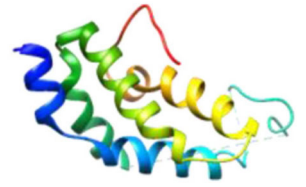
Ara h 1  
(3S7I)



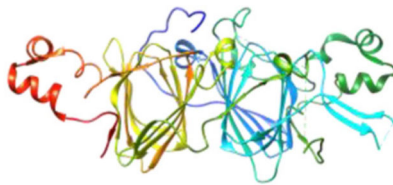
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(3SMH)



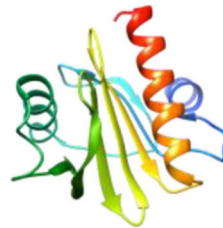
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(3OB4)



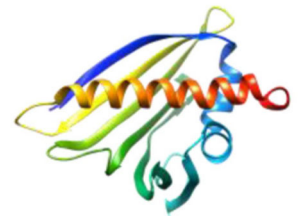
Ara h 3  
(3C3V)



Ara h 5  
(4ESP)



Ara h 8  
(4M9B)



**Fig. 1.** Ribbon representations of the known structures of peanut allergens. The Protein Data Bank accession numbers are given in brackets.



**Table 1**  
**Peanut allergen sequences and accession numbers.**

Allergen	MW	Genbank Nucleotide	GenBank Protein	Reference
Ara h 1.0101 = Ara h 1 clone 41B	69 kDa	L34402	AAB00861	Burks et al. (1995) and Viquez et al. (2003)
Ara h 1 clone P17	66 kDa	L38853	AAA60336	Burks et al. (1995)
Ara h 2.0101	16.65 kDa	L77197	AAK96887	Stanley et al. (1997) and Viquez et al. (2001)
Ara h 2.0201	18.05 kDa	AY158467	AAN77576	Chatel et al. (2003)
Ara h 3.0101	61 kDa	AF093541	AAC63045	Rabjohn et al. (1999)
Ara h 3.0201	57 kDa	AF086821	AAD47382	Kleber-Janke et al. (1999)
Ara h 3 genomic clone	61.7 kDa	AF510854	AAM46958	Viquez et al. (2004)
iso-Ara h 3	60 kDa	AY618460	AAT39430	Boldt et al. (2005)
Ara h 5.0101	15 kDa	AF059616	AAD55587	Kleber-Janke et al. (1999) and Kleber-Janke et al. (2001)
Ara h 6.0101	14.5 kDa	AF092846	AAD56337	Kleber-Janke et al. (1999)
Ara h 7.0101	16.322 kDa	AF091737	AAD56719	Kleber-Janke et al. (1999)
Ara h 7.0201	17.374	EU046325	ABW17159	Schmidt et al. (2009)
Ara h 7.0301	17.3 kDa	AY722691	AAU21496	Yan et al. (2005)
Ara h 8.0101	16.9 kDa	AY328088	AAQ91847	Mittag et al. (2004)
Ara h 8.0201	16.9 kDa	EF436550	ABP97433	Riecken et al. (2008)
Ara h 9.0101	9.13 kDa	EU159429	ABX56711	Krause et al. (2009)
Ara h 9.0201	9.04 kDa	EU161278	ABX75045	Krause et al. (2009)
Ara h 10.0101 = oleosin 1	16 kDa	AY722694	AAU21499	Schwager et al. (2015)
Ara h 10.0102	16 kDa	AY722695	AAU21500	Schwager et al. (2015)
Ara h 11.0101	14 kDa	DQ097716	AAZ20276	Schwager et al. (2015)
Ara h 11.0102 = oleosin 2	14 kDa	DQ097717	AAZ20277	Schwager et al. (2015)
Ara h 12.0101	5.2 kDa	EY396089	n/a	Petersen et al. (2015)
Ara h 13.0101	5.5 kDa	EY396019	n/a	Petersen et al. (2015)
Ara h 13.0102	5.5 kDa	EE124955	n/a	Petersen et al. (2015)
Ara h 14.0101 = oleosin variant A	18.4 kDa	AF325917	AAK13449	Pons et al. (2005) and Schwager et al. (2015)
Ara h 14.0102 = oleosin variant B	18.4 kDa	AF325918	AAK13450	Pons et al. (2005) and Schwager et al. (2015)
Ara h 14.0103 = oleosin 5	18.4 kDa	AY605694	AAT11925	Schwager et al. (2015)
Ara h 15.0101 = oleosin 3	17 kDa	AY722696	AAU21501	Schwager et al. (2015)
Ara h 16.0101	8.5 kDa	n/a	n/a	n/a
Ara h 17.0101	11 kDa	n/a	n/a	n/a

**Table 2**  
**Production of recombinant peanut allergens.**

Allergen	Host organism	Plasmid	Yield	Reference
Ara h 1	<i>E. coli</i> BL21(DE3)	pET-9a	n/a	Hurlburt et al. (2014)
Ara h 2.01	<i>E. coli</i> BL21(DE3)-Codon Plus RIL	pET-16b	Soluble fraction 4.5 mg/L, inclusion bodies 24 mg/L	Kleber-Janke and Becker (2000)
Ara h 2.01	<i>E. coli</i> Origami (DE3)	pET-32a	19 mg/L	Lehmann et al. (2003)
Ara h 2.01	<i>E. coli</i> Origami B	pMALX_E	n/a	Mueller et al. (2011)
Ara h 2.02	<i>E. coli</i> BL21 (DE3)	pET-28a(+)	74 mg/L	Lew and Lim (2016)
Ara h 2.02	<i>Lactococcus lactis</i>	pAMJ399	40 mg/L	Glenting et al. (2007)
Ara h 2.02	<i>Chlamydomonas reinhardtii</i>	pJAG15	n/a	Mayfield et al. (2003)
Ara h 5	<i>E. coli</i> Rosetta2(DE3)	pET-21d	29 mg/L	Cabanos et al. (2010a)
Ara h 6	<i>Pichia pastoris PichiaPink</i>	pPink-HC	n/a	Zhuang et al. (2012)
Ara h 10	<i>Spodoptera frugiperda</i> Sf9	pENTR4	0.9 mg/L	Cabanos et al. (2011a)
Ara h 11	<i>Spodoptera frugiperda</i> Sf9	pENTR4	0.8 mg/L	Cabanos et al. (2011a)
Ara h 14	<i>Spodoptera frugiperda</i> Sf9	pENTR4	1.3 mg/L	Cabanos et al. (2011a)

Table 3

## Purification of natural peanut allergens.

Allergen	Purification protocol	Purity	Yield	Reference
Ara h 1	Source: Virginia Extraction buffer: water Ammonium sulfate fractionation ConA affinity chromatography	Assessed by RP-HPLC	n/a	Eiwegger et al. (2006)
Ara h 1	Extraction buffer: water Ammonium sulfate fractionation ConA affinity chromatography (HPLC) SEC (HPLC)	n/a	n/a	Marsh et al. (2008)
Ara h 2	Source: Runner Extraction buffer: 20 mM sodium phosphate buffer, pH 7.0 IEC (FPLC) 2-D electrophoresis	n/a	n/a	Burks et al. (1992)
Ara h 2	Extraction buffer: 50 mM Tris/HCl, pH 8.2 Ammonium sulfate fractionation SEC IEC (HPLC) RP-HPLC	n/a	n/a	Marsh et al. (2008)
Ara h 2	Extraction buffer: 50 mM Tris, 200 mM NaCl, pH 8.3, Ammonium sulfate fractionation (40-50%) Ultrafiltration	100%	0.2 mg (7.1%)	Masuyama et al. (2014)
Ara h 3	Source: Runner Extraction buffer: 20 mM Tris, pH 7.2 IEC (FPLC) SEC	n/a	n/a	Koppelman et al. (2003)
Ara h 6	Extraction buffer: 0.1 M Ammonium carbonate, pH 8.0 SEC (HPLC) IEC (HPLC)	n/a	n/a	Suhr et al. (2004)
Ara h 6	Extraction buffer: 50 mM Tris/HCl, pH 8.2 Ammonium sulfate fractionation SEC IEC (HPLC) RP-HPLC	n/a	n/a	Marsh et al. (2008)
Ara h 7	Extraction buffer: 0.1 M ammonium bicarbonate, pH 8.0 SEC (HPLC) 2-D Electrophoresis	n/a	n/a	Schmidt et al. (2010)
Ara h 8	Extraction buffer: 0.1 M ammonium acetate, pH 5.0 SEC (HPLC) IEC (HPLC)	n/a	n/a	Riecken et al. (2008)
Ara h 8	Extraction buffer: 50 mM sodium carbonate buffer, pH 11 at 25°C Chloroform/methanol (v/v = 5/4) HIC (FPLC) SEC (FPLC)	n/a	20 µg from 1 g of unroasted peanut flour; 8 µg from 1 g of roasted peanut flour	Petersen et al. (2014)

Allergen	Purification protocol	Purity	Yield	Reference
Ara h 9	Extraction buffer: 0.1 M Tris/HCl, 10 mM EDTA, pH 7.5 followed by extraction with distilled H <sub>2</sub> O. The pellet was then extracted with 1.5 M LiCl at 4 °C for 1 h IEC (FPLC) RP <sub>2</sub> -(HPLC)	Purity 95%	1.3 mg from 110 g peanuts (0.16 %)	Lauer et al. (2009)
Ara h 10, 11, 14, 15	Extraction buffer: 0.1 M potassium phosphate, 0.33 M sucrose, pH 7.2; Isolation and delipidation of oilbodies Preparative electrophoresis	n/a	n/a	Schwager et al. (2015)
Ara h 12, 13	Extraction buffer: 50 mM sodium carbonate buffer, pH 11 at 25 °C Chloroform/methanol (v/v = 5/4) HIC (FPLC) SEC (FPLC) IEC (FPLC)	n/a	n/a	Petersen et al. (2015)

**Table 4**  
**Crystal structures of peanut allergens.**

Allergen	Method	Resolution	Protein family	PDB accession number	Reference
rAra h 1 (aa 170-586)	X-ray crystallography	2.71 Å, 2.35 Å	Vicilin, cupin superfamily	3S7E, 3S7I	Chruszcz et al. (2011)
rAra h 1 (aa 145-562)	X-ray crystallography	2.433 Å	Vicilin, cupin superfamily	3SMH	Cabanos et al. (2010b) and Cabanos et al. (2011b)
rMBP-Ara h 2	X-ray crystallography	2.706 Å	2S albumin	3OB4	Mueller et al. (2011)
nAra h 3	X-ray crystallography	1.73 Å	Legumin, cupin superfamily	3C3V	Jin et al. (2007) and Jin et al. (2009)
rAra h 5	X-ray crystallography	1.10 Å	Profilin	4ESP	Wang et al. (2013)
rAra h 8	X-ray crystallography	1.60 Å	Bet v 1	4M9B	Hurlburt et al. (2013)

**Table 5**  
**Recombinant hypoallergenic derivatives of peanut allergens.**

Allergen	Study approach	Effects	Reference
Ara h 1, 2, 3	IgE from peanut-allergic patients, T cell proliferation assay, Murine model of peanut anaphylaxis	↓ IgE-binding capacity retention of T cell reactivity	Bannon et al. (2001)
Ara h 2	RBL-2H3 cells and PMBCs derived from peanut allergic individuals	↓ IgE-binding capacity, retention of T cell reactivity	King et al. (2005)
Ara h 3	IgE from peanut-allergic patients, T cell proliferation assay	↓ IgE-binding capacity, retention of T cell reactivity	Rabjohn et al. (2002)
Ara h 1, 2, 3	Modified peanut allergens Ara h 1-3 administered subcutaneously with heat-killed <i>Listeria monocytogenes</i> in a mouse model of hypersensitivity	↓ Peanut-specific IgE ↓ Histamine levels in the plasma ↓ Anaphylaxis ↓ Th2 response	Li et al. (2003)

**Table 6**  
**Clinical trials for immunotherapy of peanut allergy.**

Approach	Study subjects and inclusion criteria	Immunizing reagent	Clinical study type	Effects	Reference
Mucosal immunotherapy	10 peanut allergic subjects, aged 18 to 50 years, with no history of severe anaphylaxis	Suspension of modified Ara h 1, 2 and 3 encapsulated in heat/phenol inactivated <i>E. coli</i>	Phase I	↓ SPT ↓ Basophil activation ↓ Systemic reactions	Wood et al. (2013)
Sublingual immunotherapy (SLIT)	40 peanut allergic subjects, aged 12 to 37 years with no history of severe anaphylaxis	Liquid peanut extract	Phase I,  Phase II safety, efficacy, and immunologic effects	↓ SPT ↑ Oral tolerance  ↑ IgG4 ↓ Side effects	Fleischer et al. (2013)
Oral exposure (Learning Early About Peanut Allergy - LEAP)	640 atopic infants, aged 4 to 11, with severe eczema and/or egg allergy	Snack food manufactured from peanut butter or smooth peanut butter	Phase II	13.7% prevalence of peanut allergy at age 60 months in the avoidance group; 1.9% in the consumption group	Du Toit et al. (2015)
Early oral immunotherapy (E-OIT)	37 children aged 9 to 36 months who had a reaction during the entry open food challenge	Peanut flour	Phase I	78% of subjects receiving E-OIT demonstrated sustained unresponsiveness 4 weeks after stopping therapy	Vickery et al. (2017)
Epicutaneous immunotherapy (EPIT)	74 peanut allergic participants aged 4 to 25 years, 25 subjects received Viaskin Peanut 100 µg (VP100), 24 subjects received Viaskin Peanut 250 µg (VP250), 25 received placebo.	Viaskin Peanut patch (DBV Technologies SA): an epicutaneous delivery system containing peanut protein extract	Phase II	Treatment success in 46% VP100 and in 48% VP250 participants; ↑ Peanut-specific IgG4 levels; ↑ IgG4/IgE ratios; ↓ basophil activation; ↓ peanut specific Th2 cytokines	Jones et al. (2017)
Epicutaneous immunotherapy (EPIT)	221 peanut allergic patients aged 6 to 55 years	Epicutaneous peanut patch containing peanut protein	Phase IIb	10-fold increase in challenge threshold of peanut protein	Sampson et al. (2017)
Peanut oral immunotherapy (OIT)	55 peanut sensitized individuals aged 4 to 26 years, with symptoms to 143 mg of peanut protein	Encapsulated defatted lightly roasted peanut flour (AR101)	Phase II for safety and efficacy	↓ Severity of DBPCFC ↑ Amount of peanut protein tolerated	Bird et al. (2018)