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(54) Title: HYPOALLERGENIC VARIANTS OF MAJOR PEANUT ALLERGEN, ARA H 2

(57) Abstract: According to an example aspect of the present invention, there is provided a modified Ara h 2 peanut allergen polypeptide, wherein at least one of the wild type DPYSPS amino acid motifs in the polypeptide comprises a first amino acid substitution at position 3 of the motif, and a second amino acid substitution at position 5 of the motif, wherein the proline (P) at position 5 of the motif is a hydroxyproline residue, wherein said modified Ara h 2 peanut allergen polypeptide is a mutant of peanut allergen polypeptide Ara h 2.0201 comprising three DPYSPS amino acid motifs, or of peanut allergen polypeptide Ara h 2.0101 comprising two DPYSPS amino acid motifs, and wherein the amino acid sequence outside said DPYSPS motifs has at least 90% sequence identity with the Ara h 2.0201 or Ara h 2.0101 sequence.



## Hypoallergenic Variants of Major Peanut Allergen, Ara h 2

### FIELD

[0001] The present invention relates to mutant polypeptides useful as hypoallergens. More specifically the present invention relates to variants of Ara h 2 proteins and the use of  
5 such polypeptides as hypoallergens for desensitizing against peanut food allergy.

### BACKGROUND

[0002] In food allergy the immune system reacts with components of food. It has been observed that the amount of human suffering of different food allergies is arising. For example, in the United States up to 8 % of young and 2-3 % of adults have allergic  
10 symptoms to food. Majority of hypersensitivity reactions are IgE-mediated (Type I allergy) (Tordesillas L et al., 2017).

[0003] Type I allergy is based on the formation of immunoglobulin E (IgE) antibodies in sensitized individuals and the symptoms occur when an allergen molecule cross-links FcεRI receptors bound IgE antibodies on the surface of effector cells such as  
15 basophils or mast cells. The cross-linking triggers the degranulation of biological mediators, such as histamine and lipid mediators that cause inflammatory reactions and symptoms, even serious systemic reactions such as anaphylaxis (Gould HJ et al., 2008).

[0004] One of the most prominent forms of IgE-mediated food allergy is peanut allergy which affects about 25 % of food allergy children in the United States. Peanut  
20 allergy is hazardous because it may often lead to serious systemic reactions, even fatal reactions. At least 16 different peanut allergen proteins have been reported. However, the recent studies have indicated that allergens Ara h 2 and Ara h 6 would be clinically the most relevant. They both are members of the 2S albumin family sharing about 60 % sequence identity which may allow cross-reactivity in immune system reactions. Most  
25 allergic individuals are sensitized for both Ara h 2 and Ara h 6, but Ara h 2 has been reported to be dominant allergen based on sensitization patterns and ability to cause allergic reactions (Hemmings O et al., 2020).

[0005] Ara h 2 is expressed in peanuts as two isoforms, Ara h 2.0101 ("short", 139 amino acids, 16,7 kDa) and Ara h 2.0201 ("long", 151 amino acids, 18,0 kDa). Ara h 2 can  
30 be proteolytically processed resulting in cleavage of the C-terminal dipeptide RY. Both

isoforms contain 8 cysteines which form 4 intramolecular disulfide bridges. In addition, Ara h 2.0101 contains two and Ara h 2.0201 three consecutive DPYSPS hexapeptide motifs in a long loop. Mass spectrometric studies have shown that in the native protein the second proline in all the motifs is hydroxylated (Hyp, O) (Li J et al., 2010).

5 [0006] The major protective mechanism of allergen immunotherapy (AIT) or desensitization operates through an induction of allergen specific “protective” or “neutralizing” IgG antibodies which can bind to wild-type allergens and compete in binding with allergen-specific IgE antibodies. This will lead to reduction of allergic symptoms and gradually to regulation of immune system towards tolerance (Linhart et al., 10 2012). The effect of allergen specific IgG antibodies were demonstrated in the recent study in which the elimination of serious systematic symptoms for peanut by using human derived specific IgG antibodies binding only to Ara h 2 allergen in a mouse model (Paolucci et al., 2023).

[0007] Current desensitization therapies are all based on the use of allergens 15 containing extracts, purified from natural sources, wherein batch to batch variations may lead to problems related to finding and maintaining the right dosage and efficiency of the treatment (Linhart et al., 2012). Today, only one desensitization therapy is available for peanut allergy, Palforzia™. It contains defatted peanut flour packed in a pill and it is consumed daily with food. Its use requires careful monitoring to follow possible serious 20 adverse reactions (Lee T et al., 2023).

[0008] The use of recombinant allergens would offer several advantages over extracts. The dose of proteins, their purity, and uniformity can be better controlled (Linhart et al., 2012). In addition, the use of pure recombinant allergens decreases the risk for neosensitization to minor allergens (Gellrich D. et al., 2020).

25 [0009] The effectiveness of recombinant allergens can be further improved by creating hypoallergenic variants in which allergic reactions triggering IgE epitopes are mutated. This allows the use of higher doses of allergen antigen to boost induction of neutralizing IgG antibodies (Linhart et al., 2012; WO2008092992). It is important to maintain the three-dimensional structure of the hypoallergen as close as possible to wild- 30 type protein structure. This kind of hypoallergen is able to induce IgG antibodies which have high affinities to wild-type allergen (Holm J et al., 2004). Therefore, a sufficient

hypoallergenicity should be achieved with a limited number of mutations which also preserve the correct fold of the protein.

[0010] The reliable information about IgE epitopes of allergens have long time been limited. However, in 2018 Croote et al. analyzed B cells from allergic individuals and determined natural Ig heavy and light-chain pairs for IgE antibodies. They identified homologous high-affinity Ara h 2 specific IgE antibodies from two individuals. This study suggests that the number of IgE epitopes is relatively low.

[0011] IgE epitopes of Ara h2 have been studied by Stanley et al (1997). They have synthesized 10-mer peptides with alanine mutations. Reduced binding to IgE was found for some mutations. However, a single alanine mutation does not necessarily change the binding affinity significantly if other residues are intact. In addition, this peptide approach did not lead to an exact identification of IgE binding hot spot residues of Ara h 2. Patent Application US20100166802 discloses similar alanine mutation approach for Ara h 2.

[0012] King et al., 2005, have prepared a variant of Ara h 2.0101 (short) by mutating 7 residues to alanine. Some alanine mutations were located in loop structures, and some of the mutants were located in the hydrophobic core of the protein.

[0013] Ramos ML et al., 2009, have studied an isoform of Ara h 2.0101 (short) which contains S73T mutation. According to our crystal structure, this mutation would not affect significantly the IgE binding.

[0014] Starkl P et al., 2012, have chemically reduced and alkylated cysteines in Ara h 2, isolated from a peanut extract. This leaves motif epitopes intact, and because nAra h 2 contains hydroxyprolines in motif epitopes, it can be estimated that preparation has still meaningful residual IgE-binding capacity and allergenicity which can be seen also in their results.

[0015] Bernard H et al., 2015, have prepared a variant for Ara h 2.0201 in which most of the long loop was deleted (residues 42-67), in addition, the variant contained mutations G40D and R41S, based on the sequence of Ara h 6. This variant showed variation in the reduction of IgE-binding as well in the mediator release capacity with different serum samples. With one serum sample, this variant showed increased mediator release potency compared to the natural Ara h 2.

[0016] Bublin et al., 2019, describe a structural variant of Ara h 2 in which the loops from the allergen surface were modified by changing the order of neighboring segments. This kind of approach may alter conformational epitopes, but linear epitopes are not changed. IgE binding and basophil activation were reduced.

5 [0017] Tscheppe A et al., 2020, have created a variant of Ara h 2.0201 in which N-terminal and C-terminal as well as the long loop were deleted. In addition, cysteines were chemically reduced and alkylated. The resulted unfolded variant had clearly reduced IgE binding and allergenicity. However, the resulted protein is structurally heterogenous and differs from the wild-type protein, consequently, its ability to induce high-affinity  
10 neutralizing antibodies is also altered.

[0018] Zhou X et al., 2023, have created three variants of Ara h 2.0201. In the first variants all 8 cysteines were mutated to glycines leading to elimination of the four disulfide bridges. The second variant includes four cysteine to glycine mutations and elimination of two disulfide bridges. In addition, it contains two tyrosines to aspartic acid mutations in the  
15 long loop. It also contains additional mutations in the second epitope. The third variant contains four cysteine mutations and tyrosine to aspartate mutations in the two loop motifs. The variants showed reduced IgE binding capacity and allergenicity, but the results do not disclose the relative contribution of different epitopes to allergenicity, or which mutations would be the most effective in allergenicity reduction. All the variants leave the third motif  
20 intact.

[0019] International patent application WO2023012652 discloses an approach in which locations of Ig-epitopes of Ara h 2.0201 were first deduced by using human derived Ara h 2 specific monoclonal antibodies. It is not mentioned if the resulted epitopes are of IgG or IgE type. Different mutation candidates were computationally generated by using  
25 the solved crystal structure (3ob4) as a template (this structure lacks most of the loop which contains the three motifs). Mutations were evaluated by computing the free energy change of protein upon mutation although it could be anticipated that the binding free energy for the immunocomplex formation should be used to estimate effect of mutations to IgE binding. Two variants were tested. B1001 variant contained 31 mutations. B764  
30 variant contained 27 mutations. Both B1001 and B764 contained mutations in the loop regions. Several mutations were located mainly on the surfaces of alpha-helices of Ara h 2. The recent crystal structure of Ara h 2 in complex with two immunotherapy-induced

neutralizing IgG antibodies (Lahood N et al., 2023) indicates that these alpha-helix regions are important for binding of neutralizing IgGs. The patent application does not reveal the most essential amino acids for IgE binding and does not lead to the selection of the most effective mutations. In addition, high number of mutations might lead to changes in the protein structure and thus diminish induction of high-affinity protective IgG antibodies capable to bind native Ara h 2.

[0020] International patent application WO2023060151 discloses Ara h 6 and Ara h 2.0101 (short) epitopes based on crystal structures of these allergens in complex with IgE Fab fragments derived from human subjects. Four epitopes were disclosed for Ara h 2: 1) N79-Q81, R113, A123-R126, D128, V131, E132; 2) Y39-P46, Q100-Q103; 3) Q33, S96-D97, L99-Q107, K109, D130-R136; 4) H27, Q30, K31-I32, R34, Q67-E68, C71, Q107, R110-E111, Q117-G120. The patent application does not disclose any mutations for claimed Ara h 2 epitopes or rank the most preferable mutant positions, substitutions, or other modifications.

## SUMMARY OF THE INVENTION

[0021] In order to identify precisely human IgE epitope for Ara h 2 allergen, we have expressed and purified PA12P3D08 (D08) antibody (Croote et al., 2018) as a recombinant Fab-fragment and the recombinant Ara h 2.0201 allergen. Said two proteins were allowed to contact with each other and an immunocomplex formed between D08 Fab fragment and Ara h 2.0201 in solution was isolated by size exclusion chromatography, crystallized and its three-dimensional structure was determined at 3.1 Å resolution by X-ray diffraction.

[0022] The crystal structure showed how three D08 Fab fragments were bound to three above mentioned DPYSPS motifs of Ara h 2.0201. The rest of the Ara h 2 structure was disordered and not visible in the electron density map. The native mass spectrum of the immunocomplex measured in solution showed the presence of the complex consisting of one full-length Ara h 2.0201 molecule and three D08 Fab molecules. The overall structure of the immunocomplex suggests that the monomeric Ara h 2.0201 molecule is very effective in cross-linking simultaneously even three D08 IgE molecules which would lead to strong cross-linking and clustering of FcεRI bound IgE antibodies on the surface of effector cells. D08 Fab is binding all three DPYSPS motifs of Ara h 2.0201 in a similar manner. The immunocomplex structure provides design of precise mutations to the IgE

epitope of Ara h 2. Our data with D08 Fab is also in agreement with earlier suggestions that posttranslational hydroxylation of the second proline in the motif DPYSPS increases the affinity of IgE binding considerably.

5 [0023] The invention is defined by the features of the independent claims. Some specific embodiments are defined in the dependent claims.

10 [0024] According to a first aspect of the present invention, there is provided a modified Ara h 2 peanut allergen polypeptide, wherein the wild type DPYSPS amino acid motifs of SEQ ID NO:1 in the polypeptide, the proline (P) at position 5 of SEQ ID NO:1 of the wild type motif being a hydroxyproline, comprise a first amino acid substitution at position 3 of SEQ ID NO:1, and a second amino acid substitution at position 5 of SEQ ID NO:1, wherein said modified Ara h 2 peanut allergen polypeptide is a mutant of peanut allergen polypeptide Ara h 2.0201 of SEQ ID NO:2 comprising three DPYSPS amino acid motifs, or of peanut allergen polypeptide Ara h 2.0101 of SEQ ID NO:3 comprising two DPYSPS amino acid motifs, and wherein the amino acid sequence of said modified polypeptide has at least 90% sequence identity with SEQ ID NO:2 or 3 outside said DPYSPS motifs, and wherein positions 1, 4, and 6 of SEQ ID NO:1 are not modified in said DPYSPS motif(s) of said modified Ara h 2 peanut allergen polypeptide, i.e. said positions 1, 4, and 6 remain intact in the modified peptide in view of the wild type allergen.

20 [0025] According to a second aspect of the present invention, there is provided a modified Ara h 2 peanut allergen polypeptide, wherein all DPYSPS (SEQ ID NO:1) amino acid motifs have been deleted, wherein said modified Ara h 2 peanut allergen polypeptide is a mutant of peanut allergen polypeptide Ara h 2.0201 of SEQ ID NO:2 comprising three DPYSPS amino acid motifs, or of peanut allergen polypeptide Ara h 2.0101 of SEQ ID NO:3 comprising two DPYSPS amino acid motifs.

25 [0026] According to a third aspect of the present invention, there is provided a pharmaceutical composition comprising the modified Ara h 2 peanut allergen polypeptide according to the present disclosure and at least one of the following: physiologically acceptable adjuvant, carrier, diluent, excipient, preservative and stabilizer.

30 [0027] According to a fourth aspect of the present invention, there is provided a method of treating peanut allergy in a subject, the method comprising: administering to the subject an amount of the modified Ara h 2 peanut allergen polypeptide according to the

present disclosure in an amount effective to ameliorate at least one symptom or clinical sign of allergy to the peanut Ara h 2 allergen, wherein the modified Ara h 2 peanut allergen polypeptide activates release of histamine from basophils to a degree less than the wild type allergen.

5   **[0028]**       According to a sixth aspect of the present invention, there is provided a method of producing a modified Ara h 2 peanut allergen polypeptide for immunotherapy comprising the steps of:

- a) modifying nucleic acid sequence encoding a wild type Ara h 2 peanut allergen polypeptide in order to substitute at least one of the wild type DPYSPS amino acid motifs of SEQ ID NO:1 in the polypeptide with a modified DPYSPS motif comprising a first  
10   amino acid substitution at position 3 of SEQ ID NO:1, and a second amino acid substitution at position 5 of SEQ ID NO:1, wherein positions 1, 4, and 6 of SEQ ID NO:1 are not modified in said modified DPYSPS motif, wherein said modified Ara h 2 peanut allergen polypeptide is preferably a mutant of peanut allergen polypeptide Ara h 2.0201 of  
15   SEQ ID NO:2 comprising three DPYSPS amino acid motifs, or of peanut allergen polypeptide Ara h 2.0101 of SEQ ID NO:3 comprising two DPYSPS amino acid motifs;
- b) expressing or producing the modified Ara h 2 peanut allergen polypeptide from the modified nucleic acid; and
- c) isolating and purifying the modified Ara h 2 peanut allergen polypeptide from step b).

20   **[0029]**       According to a seventh aspect of the present invention, there is provided an isolated nucleic acid sequence encoding the modified Ara h 2 peanut allergen polypeptide according to the present disclosure.

**[0030]**       According to an eighth aspect of the present invention, there is provided an isolated vector comprising a nucleic acid sequence encoding the modified Ara h 2 peanut  
25   allergen polypeptide according to the present disclosure.

**[0031]**       According to a ninth aspect of the present invention, there is provided an isolated host cell comprising a vector comprising a nucleic acid sequence encoding the modified Ara h 2 peanut allergen polypeptide according to the present disclosure.

## BRIEF DESCRIPTION OF THE DRAWINGS

- [0032] FIGURE 1 illustrates the crystal structure of Ara h 2.0201 in complex with three D08 IgE Fab fragments. Only the loop containing the three DPYSPS motifs was visible in the electron density for the crystal structure refinement. The rest of the Ara h 2 structure was disordered and modelled (A). (B) shows the binding of the PYSPS motif to the binding site of D08 antibody. O is referring to hydroxyproline in the native Ara h 2.
- [0033] FIGURE 2. Results of competitive immunoassay measuring the inhibition of IgE binding of a serum pool to immobilized natural Ara h 2 in the presence of increasing soluble concentrations of natural Ara h 2 (nA2) and Ara h 2 variants 1-5 (A2v1, A2v2, A2v3, A2v4 and A2v5).
- [0034] FIGURE 3. Results of competitive immunoassay measuring the inhibition of IgE binding of nine individual sera (A-I) to immobilized natural Ara h 2 in the presence of increasing soluble concentrations of natural Ara h 2 (nA2) and Ara h 2 variants 1 and 5 (A2v1 and A2v5).
- [0035] FIGURE 4. Histamine release induced by natural Ara h 2 (nA2), Ara h 2 variants v1 and v5. Released histamine was measured after the passive sensitization of stripped basophils with sera of three peanut allergic persons (A, B, C).
- [0036] FIGURE 5. Results of the competitive immunoassay measuring the inhibition of IgE binding of a serum pool to immobilized natural Ara h 2 in the presence of increasing concentrations of soluble natural Ara h 2 (nA2) and recombinant Ara h 2 variants rA2v4, rA2v6 and rA2v8.
- [0037] FIGURE 6. Results of the competitive immunoassay measuring the inhibition of IgE binding of nine individual sera (A – G) to immobilized natural Ara h 2 in the presence of increasing concentrations of soluble natural Ara h 2 (nA2) and recombinant Ara h 2 variants rA2v4, rA2v6 and rA2v8.
- [0038] FIGURE 7. Histamine release induced by natural Ara h 2 (nA2), Ara h 2 variants v4 and v6. Released histamine was measured after the passive sensitization of stripped basophils with sera of two peanut allergic persons (A, B).
- [0039] FIGURE 8. Analysis of the expression levels of Ara h 2 variant 6 and Ara h 2 variant B1001 (disclosed in WO 2023/012652) in BL21 *E.coli* strain using the expression

vector pET28b+. Lane 1: MW marker, lane 2: purified Ara h 2 variant 6 (MW 16690 Da) control, lane 3: cell lysate of Ara h 6 variant, lane 4: purified Ara h 2.0201 control (MW 18065 Da), lanes 5-8 cell lysates isolated from four different expression clones of B1001 (MW 17520 Da).

- 5    **[0040]**        FIGURE 9. The crystal structure of human IgE based D08 Fab in complex with rAra h 2.0201 shows the binding of the five amino acid DPYSP peptide of the DPYSPS motif to the paratope of the Fab. The DPYSP peptide is shown in the middle. The residues of the motif peptide are numbered. The residue numbers in brackets are for the first motif of Ara h 2.0201. It is notable that the residue of 1 (D) is not in contact with the Fab, but residue 2 (P) is in contact with the Fab.
- 10

## EMBODIMENTS

- [0041]**        The modified hypoallergenic polypeptides according to the present disclosure have preferably a structure which as closely as possible reflects the wild type amino acid sequence of the allergen, but additionally contains amino acid substitutions at selected amino acid positions to reduce or fully diminish their ability to bind to IgE antibodies. It is important that the modified polypeptides still retain their capacity to induce the production of protective IgG antibodies which are binding also the corresponding folded wild type allergen.
- 15

- [0042]**        A person skilled in the art knows that nucleotide mutations leading to amino acid substitutions, additions and/or deletions at “non-essential” amino acid residues can also be made to the sequence of the wild type allergen, but this is not a particular aim of the present invention although the modified polypeptides of the present disclosure may also comprise non-essential mutations, elongations, insertions and deletions in addition to the substitutions providing the effect of the present invention. A “non-essential” amino acid residue is a residue that can be modified in the wild-type sequences of the allergen polypeptide without altering its biological activity or three-dimensional structure. Amino acids for which conservative substitutions can be made are well known in the art.
- 20
- 25

- [0043]**        Further mutations conserving the biological activity but giving slightly modified physical properties, such as increased solubility, can also be introduced to the hypoallergenic polypeptide, see e.g. Trevino et al., 2007, Journal of Molecular Biology
- 30

366:449-460, disclosing amino acid modifications contributing to solubility of the modified protein.

**[0044]** In the present description, examples, claims and sequence listing both three-letter and one-letter codes may be used for amino acids. See, for instance, IUPAC-IUB  
5 Joint Commission on Biochemical Nomenclature. Nomenclature and Symbolism for Amino Acids and Peptides. Eur. J. Biochem. 138:9-37(1984). The denomination of amino acid sites in the polypeptides according to the present disclosure are exemplified as follows: P43 means that there is a proline residue at position 43, whereas P43D means that the proline residue at position 43 has been replaced by an aspartic acid residue.

10 **[0045]** Peanut allergy is a very common form of food allergy which often leads to systemic reactions, even anaphylaxis. According to the literature, Ara h 2 exists in two highly homologues major forms Ara h 2.0101 and Ara h 2.0202 and is clinically the most relevant peanut allergen. It is responsible for the IgE binding in more than 90 % of peanut allergic subjects and it is considered the most anaphylactic. Ara h 2.0101 has 139 residues  
15 (16.7 kDa), and Ara h 2.0202 has 151 residues (18 kDa). They both have eight cysteines which form four intramolecular disulfide bridges which leads to the formation of very stable fold. The major difference between Ara h 2.0101 and Ara h 2.0201 is the difference in the number of DPYSPS motif repeats in a long loop in the middle of the polypeptide chain. Ara h 2.0101 contains two repeats and Ara h 2.0201 three repeats. The crystal  
20 structure of immunocomplex structure of Ara h 2.0201 with three D08 Fabs shows how the monomeric allergen is able to cross-link at the same time even three IgE antibodies using a three motif containing loop (see Figure 1). This leads to very efficient cross-linking of FcεRI receptor bound IgE antibodies on the surface of the effector cell and subsequent triggering of allergic reactions. Because the conformation of motifs containing loop is not  
25 depending on the correct fold of the core protein it is evident that the unfolded or denaturated Ara h 2 has still ability to cross-link IgE antibodies.

**[0046]** The importance of DPYSPS motif for IgE binding has been well documented in the literature. However, the finding that monomeric Ara h 2 would be able to bind simultaneously to three IgE antibodies has not been described earlier. Although mutational  
30 studies have earlier been done in motifs, the lack of experimental three-dimensional structure for Ara h 2 in complex with genuine IgE antibody has not allowed the

identification of key residues of IgE binding and design of effective hypoallergenic variants.

[0047] The present invention provides mutated hypoallergenic variants of Ara h 2, which are useful as vaccines or immunotherapy agents for immunizing subjects in need thereof and thus preventing and/or alleviating allergy and desensitizing subjects suffering from allergy against peanut.

[0048] Specifically, the present invention provides a modified Ara h 2 peanut allergen polypeptide, wherein at least one of the wild type DPYSPS amino acid motifs of SEQ ID NO:1 in the polypeptide comprises a first amino acid substitution at position 3 of SEQ ID NO:1, and a second amino acid substitution at position 5 of SEQ ID NO:1, wherein the proline (P) at position 5 of SEQ ID NO:1 in the wild type allergen is a hydroxyproline residue (i.e. the motif has the structure DPYSP<sup>OH</sup>S), wherein said modified Ara h 2 peanut allergen polypeptide is a mutant of peanut allergen polypeptide Ara h 2.0201 of SEQ ID NO:2 comprising three DPYSPS amino acid motifs, or of peanut allergen polypeptide Ara h 2.0101 of SEQ ID NO:3 comprising two DPYSPS amino acid motifs, and wherein the amino acid sequence of said modified polypeptide has at least 90% sequence identity with SEQ ID NO:2 or 3 outside said DPYSPS motifs, and wherein positions 1, 4, and 6 of SEQ ID NO:1 are not modified in said DPYSPS motif(s) of said modified Ara h 2 peanut allergen polypeptide, i.e., said positions 1, 4, and 6 remain intact in the modified peptide.

[0049] The polypeptides according to the present invention are hypoallergenic, and exhibit a histamine release capacity when compared to the histamine release capacity of the unmutated native Ara h 2 wild type (with hydroxyprolines).

[0050] The hypoallergenic polypeptides according to the present invention are useful as immunotherapy agents against peanut allergy. AIT preparations comprising polypeptides according to the present invention are formulated according to standard pharmaceutical procedures known to skilled persons in the art. Vaccine-like preparations according to the present invention are especially suited for subcutaneous administration. For instance, a hypoallergen according to the present invention is formulated as conventional vaccine formulations, such as aluminum hydroxide adsorbed vaccines, using methods well known in the art (Niederberger et al., PNAS, 101 (2): 14677-82, 2004). Alternatively, and preferably, however, the modified polypeptides according to the present

invention may be administered by other suitable AIT routes and schemes, such as oromucosal (oral) or sublingual administration, using methods and formulations known in the art. See, e.g., European Patent publication EP 1812059.

[0051] The determined crystal structure of the immunocomplex showed that the first residue in the motif, aspartate (D) did not interact with the antibody. The crystal structure of the immunocomplex was used as a template to test all possible 19 mutations in the remaining five residues (PYSPS). When mutating residues computationally, the effect of mutations was evaluated on the basis of favourable or unfavourable interactions with D08 Fab. Unfavourable interactions would include sterical collisions, hydrophobic-hydrophilic interactions, or formation of unfavourable charge interactions (positive-positive or negative-negative). In this case unfavourable interactions are targeted because these would decrease binding affinity to D08 IgE antibody. We assumed that the effect of mutations is similar in all three motifs in Ara h 2.0201 and in all two motifs of Ara h 2.0101. The results from this study are presented in Table 1.

[0052] **Table 1.** Estimation of the effects of mutations in Ara h 2 PYSPS motifs based on the immunocomplex structure of Ara h 2.0201 and D08 Fab. +: decreases Ara h 2 binding to IgE (preferable); -: increases binding (not preferable); 0: no effect (not preferable); O, hydroxyproline; na, not applicable. The mutants utilized in the variants disclosed below in the Experimental Section are in bold.

20

| Ara h 2.0201 (long), SEQ ID NO:2  |     |     |     |     |     |
|-----------------------------------|-----|-----|-----|-----|-----|
| motif 1                           | P43 | Y44 | S45 | O46 | S47 |
| motif 2                           | P50 | Y51 | S52 | O53 | S54 |
| motif 3                           | P62 | Y63 | S64 | O65 | S66 |
| Ara h 2.0101 (short), SEQ ID NO:3 |     |     |     |     |     |
| motif 1                           | P43 | Y44 | S45 | O46 | S47 |
| motif 2                           | P50 | Y51 | S52 | O53 | S54 |
|                                   |     |     |     |     |     |
| mutation N                        | +   | ++  | 0   | +   | 0   |
| mutation C                        | +   | +   | 0   | +   | 0   |

|            |    |     |    |     |    |
|------------|----|-----|----|-----|----|
| mutation Q | +  | +   | 0  | +   | 0  |
| mutation H | +  | 0   | 0  | ++  | +  |
| mutation S | +  | +   | na | +   | na |
| mutation T | +  | +   | 0  | +   | 0  |
| mutation K | -  | ++  | 0  | ++  | 0  |
| mutation R | 0  | ++  | 0  | ++  | 0  |
| mutation D | ++ | +++ | 0  | +++ | 0  |
| mutation E | ++ | +++ | 0  | +++ | 0  |
| mutation Y | ++ | na  | +  | +++ | 0  |
| mutation W | ++ | +++ | -  | +++ | +  |
| mutation F | ++ | 0   | +  | +++ | 0  |
| mutation A | 0  | +   | 0  | +   | 0  |
| mutation V | +  | 0   | 0  | +   | 0  |
| mutation L | ++ | 0   | 0  | +++ | 0  |
| mutation I | ++ | +   | 0  | +++ | 0  |
| mutation M | +  | 0   | 0  | +++ | 0  |
| mutation G | 0  | +   | 0  | +   | 0  |
| mutation P | na | +   | +  | +   | +  |

**[0053]** The modified hypoallergens of the present disclosure could be administered in concentrations of, e.g., 0.5 µg/ml, 5 µg/ml or 50 µg/ml. Exemplary doses may vary between 0.05 µg and 2 µg during a possible dosing-up phase, and between 3-15 µg during the maintenance phase, preferably 5-15 µg, most preferably about 10 µg, depending on the severity of the allergy, the age and medical history of the patient. A suitable dose is easily decided by a clinician familiar with treating and preventing allergy.

**[0054]** International patent publication WO2004047794 discloses a solid fast dispersing dosage form for sublingual administration of an allergy vaccine, and US patent application 20090297564 discloses a liquid vaccine formulation for oromucosal administration.

[0055] The hypoallergenic variant polypeptides according to the present invention, useful in allergen-specific desensitization, possess two features: 1) the ability to strongly reduce an IgE-mediated reaction; and 2) a retained wild-type 3D folding, and thus the capability of inducing the production of IgG-antibodies capable to bind wt allergen with high affinity.

[0056] In a preferred embodiment, each of said two or three DPYSPS amino acid motifs in said modified Ara h 2 peanut allergen polypeptides comprises a first amino acid substitution at position 3 of SEQ ID NO:1, and a second amino acid substitution at position 5 of SEQ ID NO:1. The modifications at positions 3 and 5 in the DPYSPS motifs in the modified polypeptide are preferably the same in all DPYSPS motifs in said modified polypeptide, but the modifications can also differ from each other in said two or three DPYSPS motifs present in the modified polypeptide.

[0057] In another preferred embodiment, positions 1, 4, and 6 of SEQ ID NO:1 are not modified in said DPYSPS motif(s).

[0058] In another preferred embodiment, at least one DPYSPS motif has been deleted.

[0059] In another preferred embodiment, at least one DPYSPS motif has been deleted in said modified Ara h 2 peanut allergen polypeptide so that said polypeptide comprises only one or two DPYSPS motif(s), wherein said remaining one or two DPYSPS motif(s) is/are modified as defined in the present disclosure.

[0060] In another preferred embodiment, said modified Ara h 2 peanut allergen polypeptide comprises only two remaining DPYSPS motifs, wherein said two DPYSPS motifs are modified as defined in the present disclosure.

[0061] In another preferred embodiment, said modified Ara h 2 peanut allergen polypeptide comprises only one remaining DPYSPS motif, wherein said DPYSPS motif is modified as defined in the present disclosures, e.g., at least positions 3 and 5 of the DPYSPS motif are modified and positions 1, 4, and 6 remain intact. In a more preferred embodiment, positions 2, 3 and 5 of the DPYSPS motif are modified and positions 1, 4, and 6 remain intact.

[0062] In another preferred embodiment, said first amino acid substitution at position 3 of SEQ ID NO:1 is from tyrosine (Y) to an amino acid residue selected from the group consisting of: glutamic acid (E), lysine (K), tryptophan (W), aspartic acid (D), asparagine (N), arginine (R), cysteine (C), glutamine (Q), serine (S), threonine (T), alanine (A), isoleucine (I), glycine (G), and proline (P).

[0063] In a more preferred embodiment, said first amino acid substitution at position 3 of SEQ ID NO:1 is from tyrosine (Y) to glutamic acid (E) or lysine (K).

[0064] In another preferred embodiment, said second amino acid substitution at position 5 of SEQ ID NO:1 is from proline (P) to an amino acid residue selected from the group consisting of: aspartic acid (D), glutamic acid (E), tyrosine (Y), tryptophan (W), phenylalanine (F), leucine (L), isoleucine (I), methionine (M), lysine (K), arginine (R), and histidine (H).

[0065] In a more preferred embodiment, said second amino acid substitution at position 5 of SEQ ID NO:1 is from proline (P) to aspartic acid (D) or glutamic acid (E).

[0066] In another preferred embodiment, each of said two or three DPYSPS amino acid motifs in said modified Ara h 2 peanut allergen polypeptides comprises a third amino acid substitution at position 2 of SEQ ID NO:1.

[0067] In a more preferred embodiment, said third amino acid substitution at position 2 of SEQ ID NO:1 is from proline (P) to an amino acid residue selected from the group consisting of: aspartic acid (D), glutamic acid (E), tyrosine (Y), tryptophan (W), phenylalanine (F), leucine (L), and isoleucine (I).

[0068] In the most preferred embodiment, said third amino acid substitution at position 2 of SEQ ID NO:1 is from proline (P) to aspartic acid (D).

[0069] In another preferred embodiment, said amino acid modifications are amino acid substitutions at positions defined by the Ara h 2.0201 wild type sequence of SEQ ID NO:2 and said positions are selected from the group consisting of positions: P43, Y44, P46, P50, Y51, P53, Y63, and P65, wherein said modified Ara h 2 peanut allergen polypeptide has an amino acid sequence having at least 90% sequence identity with the amino acid sequence of SEQ ID NO:2 outside the DPYSPS motifs (i.e. the sequence outside the DPYSPS motif(s) in the modified polypeptide has at least 90% sequence

identity with the amino acid sequence outside the DPYSPS motifs of SEQ ID NO:2). More preferably, said sequence identity is 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%. As discussed above, the aim of the present disclosure is to mutate or delete the DPYSPS motifs and leave the sequences outside said DPYSPS motif(s) as intact as possible in order to induce patient's IgG antibodies which have high affinities to wild-type allergen and thus provide the hypoallergenic effect with the modified polypeptide of the present disclosure.

[0070] In another preferred embodiment, said amino acid modifications are selected from the group consisting of: P43D, Y44E, Y44K, P46D, P46E, P50D, Y51E, Y51K, P53D, P53E, P62D, Y63E, Y63K, P65D, and P65E.

[0071] In another preferred embodiment, said amino acid modifications are Y44E, P46D, Y51E, P53D, Y63E, and P65D.

[0072] In another preferred embodiment, said amino acid modifications are Y44E, P46E, Y51E, P53E, Y63E, and P65E.

[0073] In another preferred embodiment, said amino acid modifications are Y44K, P46D, Y51K, P53D, Y63K, and P65D.

[0074] In another preferred embodiment, said amino acid modifications are P43D, Y44K, P46D, P50D, Y51K, P53D, P62D, Y63K, and P65D.

[0075] In another preferred embodiment, at least one DPYSPS (SEQ ID NO:1) amino acid motifs in the polypeptide have been deleted, wherein said modified Ara h 2 peanut allergen polypeptide is a mutant of peanut allergen polypeptide Ara h 2.0201 of SEQ ID NO:2 comprising three DPYSPS amino acid motifs, or of peanut allergen polypeptide Ara h 2.0101 of SEQ ID NO:3 comprising two DPYSPS amino acid motifs, and wherein remaining DPYSPS (SEQ ID NO:1) amino acid motif(s) has/have been modified so that the motif comprises a first amino acid substitution at position 3 of SEQ ID NO:1, and a second amino acid substitution at position 5 of SEQ ID NO:1.

[0076] In another preferred embodiment, the amino acid at position P67 or Y68 defined by the Ara h 2.0201 wild type sequence of SEQ ID NO:2 comprises an amino acid substitution, preferably said amino acid modification is P67A or Y68D, respectively.

[0077] The present invention also provides a modified Ara h 2 peanut allergen polypeptide, wherein all DPYSPS (SEQ ID NO:1) amino acid motifs have been deleted, wherein said modified Ara h 2 peanut allergen polypeptide is a mutant of peanut allergen polypeptide Ara h 2.0201 of SEQ ID NO:2 comprising three DPYSPS amino acid motifs, or of peanut allergen polypeptide Ara h 2.0101 of SEQ ID NO:3 comprising two DPYSPS amino acid motifs.

[0078] In a preferred embodiment, said deleted area in the polypeptide corresponds to positions 41-66 of the Ara h 2.0201 wild type sequence of SEQ ID NO:2.

[0079] In another preferred embodiment, said modified Ara h 2 peanut allergen polypeptide has an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:2 outside the DPYSPS motifs at positions between R41-S66 and P67/Y68 of SEQ ID NO:2, or at least 90% sequence identity to the amino acid sequence of SEQ ID NO:3 outside the DPYSPS motifs at positions between R41-S54 of SEQ ID NO:3. More preferably, said sequence identity is 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%.

[0080] In another preferred embodiment, the amino acid at position P67 defined by the Ara h 2.0201 wild type sequence of SEQ ID NO:2 comprises an amino acid substitution, preferably said amino acid modification is P67A, and or the amino acid at position Y68 defined by the Ara h 2.0201 wild type sequence of SEQ ID NO:2 comprises an amino acid substitution, preferably said amino acid modification is Y68D.

[0081] In another preferred embodiment, the modified Ara h 2 peanut allergen polypeptide activates release of histamine from basophils to a degree less than a corresponding wild type allergen.

[0082] The present invention is also directed to a pharmaceutical composition comprising the modified Ara h 2 peanut allergen polypeptide of the present disclosure and at least one of the following: physiologically acceptable adjuvant, carrier, diluent, excipient, preservative and stabilizer.

[0083] In a preferred embodiment, said composition is a vaccine composition comprising a pharmaceutically acceptable diluent or adjuvant.

[0084] The present invention is further directed to a method of treating peanut allergy in a subject, the method comprising: administering to the subject an amount of the modified Ara h 2 peanut allergen polypeptide according to the present disclosure in an amount effective to ameliorate at least one symptom or clinical sign of allergy to the peanut Ara h 2 allergen, wherein the modified Ara h 2 peanut allergen polypeptide activates release of histamine from basophils to a degree less than the wild type allergen.

[0085] The present invention also provides a method of producing a modified Ara h 2 peanut allergen polypeptide for immunotherapy comprising the steps of:

a) modifying nucleic acid sequence encoding a wild type Ara h 2 peanut allergen polypeptide in order to substitute at least one of the wild type DPYSPS amino acid motifs of SEQ ID NO:1 in the polypeptide with a modified DPYSPS motif comprising a first amino acid substitution at position 3 of SEQ ID NO:1, and a second amino acid substitution at position 5 of SEQ ID NO:1, and wherein positions 1, 4, and 6 of SEQ ID NO:1 are not modified in said modified DPYSPS motif, wherein said modified Ara h 2 peanut allergen polypeptide is preferably a mutant of peanut allergen polypeptide Ara h 2.0201 of SEQ ID NO:2 comprising three DPYSPS amino acid motifs, or of peanut allergen polypeptide Ara h 2.0101 of SEQ ID NO:3 comprising two DPYSPS amino acid motifs;

b) expressing or producing the modified Ara h 2 peanut allergen polypeptide from the modified nucleic acid; and

c) isolating and purifying the modified Ara h 2 peanut allergen polypeptide from step b).

[0086] In a preferred embodiment, said method comprises a further step of: d) testing the modified Ara h 2 peanut allergen polypeptide obtained from step b) or c) for ability to activate release of histamine of an allergic reaction from human cells, wherein those the modified Ara h 2 peanut allergen polypeptides which do not activate histamine release or which activate histamine release less than the corresponding wild type Ara h 2 polypeptide are considered as hypoallergen candidates for immunotherapy.

[0087] In another preferred embodiment, said method comprises a step of: e) analyzing that said modified Ara h 2 peanut allergen polypeptide has a native-like structure and immunogenic potential to develop protective IgG antibodies.

[0088] In another preferred embodiment, in said method the nucleic acid sequence encoding a wild type Ara h 2 polypeptide is modified so that the amino acid substitutions formed are at positions defined by the Ara h 2.0201 wild type sequence of SEQ ID NO:2 and said substitutions are selected from the group consisting of: P43D, Y44E, Y44K, 5 P46D, P46E, P50D, Y51E, Y51K, P53D, P53E, P62D, Y63E, Y63K, P65D, and P65E.

[0089] In another preferred embodiment, said amino acid substitutions are i) Y44E, P46D, Y51E, P53D, Y63E, and P65D; ii) Y44E, P46E, Y51E, P53E, Y63E, and P65E; iii) Y44K, P46D, Y51K, P53D, Y63K, and P65D; or iv) P43D, Y44K, P46D, P50D, Y51K, P53D, P62D, Y63K, and P65D.

10 [0090] The present invention is also directed to an isolated nucleic acid sequence encoding the modified Ara h 2 peanut allergen polypeptide according to the present disclosure, an isolated vector comprising a nucleic acid sequence encoding the modified Ara h 2 peanut allergen polypeptide according to the present disclosure, as well as to an isolated host cell comprising a vector comprising a nucleic acid sequence encoding the 15 modified Ara h 2 peanut allergen polypeptide according to the present disclosure.

[0091] It is to be understood that the embodiments of the invention disclosed are not limited to the particular structures, process steps, or materials disclosed herein, but are extended to equivalents thereof as would be recognized by those ordinarily skilled in the relevant arts. It should also be understood that terminology employed herein is used for the 20 purpose of describing particular embodiments only and is not intended to be limiting.

[0092] Reference throughout this specification to “one embodiment” or “an embodiment” means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases “in one embodiment” or “in an embodiment” 25 in various places throughout this specification are not necessarily all referring to the same embodiment.

[0093] As used herein, a plurality of items, structural elements, compositional elements, and/or materials may be presented in a common list for convenience. However, these lists should be construed as though each member of the list is individually identified 30 as a separate and unique member. Thus, no individual member of such list should be construed as a de facto equivalent of any other member of the same list solely based on their presentation in a common group without indications to the contrary. In addition,

various embodiments and example of the present invention may be referred to herein along with alternatives for the various components thereof. It is understood that such embodiments, examples, and alternatives are not to be construed as de facto equivalents of one another, but are to be considered as separate and autonomous representations of the present invention.

[0094] Furthermore, the described features, structures, or characteristics may be combined in any suitable manner in one or more embodiments. In the following description, numerous specific details are provided, such as examples of lengths, widths, shapes, etc., to provide a thorough understanding of embodiments of the invention. One skilled in the relevant art will recognize, however, that the invention can be practiced without one or more of the specific details, or with other methods, components, materials, etc. In other instances, well-known structures, materials, or operations are not shown or described in detail to avoid obscuring aspects of the invention.

[0095] While the forgoing examples are illustrative of the principles of the present invention in one or more particular applications, it will be apparent to those of ordinary skill in the art that numerous modifications in form, usage and details of implementation can be made without the exercise of inventive faculty, and without departing from the principles and concepts of the invention. Accordingly, it is not intended that the invention be limited, except as by the claims set forth below.

[0096] The verbs “to comprise” and “to include” are used in this document as open limitations that neither exclude nor require the existence of also un-recited features. The features recited in depending on claims are mutually freely combinable unless otherwise explicitly stated. Furthermore, it is to be understood that the use of "a" or "an", i.e. a singular form, throughout this document does not exclude a plurality.

## EXPERIMENTAL SECTION

EXAMPLE 1. **Production and purification of recombinant Ara h 2 polypeptides**

Synthetic gene fragments encoding Ara h 2.0101 (SEQ ID NO:3) and Ara h 2.0201 (SEQ ID NO:2), A2v1 (SEQ ID NO:4), A2v2 (SEQ ID NO:5), A2v3 (SEQ ID NO:6), A2v4 (SEQ ID NO:7), A2v5 (SEQ ID NO:8), A2v6 (SEQ ID NO:9), A2v7 (SEQ ID NO:10) and A2v8 (SEQ ID NO:11) with codon optimization for *E.coli* expression were purchased from GeneScript. The amino acid sequences of A2v1, A2v2, A2v3, A2v4, A2v5, A2v6, A2v7 and A2v8 are shown in the Sequence Listing as SEQ ID NOS:4-11, respectively. The synthetic gene fragments of Ara h 2.0101, Ara h 2.0201 and mutants A2v1-A2v8 were cloned as Nco I-Not I restriction fragments under the T7 promoter of the pET-28b(+) vector (Novagen) for cytoplasmic expression. The recombinant Ara h 2 allergens contain an extra alanine residue in the NH<sub>2</sub>-terminus due the usage of Nco I restriction site in the cloning of the synthetic genes. The Nco I cleavage site sequence (CCATGG) introduces the ATG codon of the methionine amino acid required for the translation initiation but also an additional G nucleotide and therefore GCC codon coding an alanine amino acid was added into the 5' sequence of the Ara h 2 allergen genes. The pET-28b expression vectors were transformed into the *E.coli* BL21 (DE3) strain. Recombinant Ara h 2 allergens were produced in 1.8 L shake flask cultivations.

Expressed Ara h 2.0101 and Ara h 2.0201 were purified from the insoluble protein fraction isolated from the cell pellets according to Hoffmann-Sommergruber et al. (1997) and refolding of the allergen polypeptides was performed according to Arango et al. (1992) and Stancombe et al. (2003). Recombinant Ara h 2.0101 and Ara h 2.0201 were purified with a two-step chromatography procedure. The Ara h 2 variants (A2v1-A2v8) were refolded and purified with a modified method. Refolding was carried out from sonicated bacterial suspensions by a multistep dialysis protocol according to Ban et al. (2020).

Recombinant Ara h 2 allergens were purified with a two-step chromatography procedure by a standard ion exchange (Ara h 2.0101 and Ara h 2.0201 with HiTrap DEAE and variants with a HiTrap Q, GE Healthcare) followed by a size-exclusion chromatography (SEC) (HiLoad Superdex 75 pg, GE Healthcare). Elution peak fractions after the final purification step were analyzed by a Coomassie-stained SDS-PAGE showing that obtained proteins were of high purity and homogeneity. Concentrations of the pure allergens were determined by measuring A<sub>280</sub> and using sequence-derived extinction coefficient.

**EXAMPLE 2. Analysis of recombinant Ara h 2 polypeptides by Mass Spectrometry**

Mass-spectrometric experiments were performed with a Bruker Solarix XR Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), equipped with an electrospray ionization (ESI) source. Denaturated  
5 spectra were typically measured in acetonitrile/water/acetic acid solution (49.5:49.5:1). Native mass spectra measurements were performed with desalted protein samples typically at concentration of 20  $\mu$ M in 10 mM ammonium acetate buffer (pH 6.9). All instrumental parameters were optimized to maintain non-covalent interactions in the gas-phase and to maximize ion transmission at m/z 2000–3000. Mass calibration was done externally with  
10 respect to the ions of an ES Tuning Mix (Agilent Technologies, Santa Clara, CA, USA).

The high-resolution denatured mass spectra of Ara h 2 allergens and variants showed that all purified proteins are homogeneous and have correct molecular weight indicating that four disulphide bridges are correctly formed. Correct folding was verified with native mass spectra analyses.

**15 EXAMPLE 3. Production and purification of the recombinant anti-Ara h 2 D08 Fab fragment**

A synthetic gene fragment encoding the Fab fragment expression unit of the anti-Ara h 2 human IgE PA12P3D08 (D08) antibody (Croote et al., 2018) was purchased from GeneScript as a human IgG1 Fab fragment. The D08 Bab expression unit was cloned into  
20 the pKKtac expression vector containing a hexahistidine tag at the C-terminus of the heavy chain constant region for IMAC-purification (Laukkanen et al., 2003; Takkinen et al., 1991). Soluble D08 fab fragments were produced in the *E.coli* RV308 strain (ATCC 31608) by a high cell density fermentation (Nevanen et al., 2001) and purified from the culture supernatant by immobilized metal affinity chromatography (IMAC) with a standard  
25 protocol. Elution peak fractions were analyzed by a Coomassie-stained 18% SDS-PAGE and fractions containing pure D08 Fab were pooled and dialyzed against PBS. The binding activity of the purified D08 Fab fragment to the recombinant Ara h 2.0201 was confirmed by ELISA.

**30 EXAMPLE 4. Isolation of the immunocomplex of D08 Fab and Ara h 2.0201 by size exclusion chromatography**

Purified D08 Fab fragment (1.5-fold molar) excess was incubated with Ara h 2.0201 in

solution for 2h at +21°C. Immunocomplex formed between D08 Fab and Ara h 2.0201 was isolated by a SEC column (HiLoad Superdex 75 pg, GE Healthcare). Elution fractions were analyzed by a Coomassie-stained 18% SDS-PAGE and fractions in which D08 Fab and Ara h 2.0201 comigrated were pooled and concentrated for crystallization.

5    **EXAMPLE 5. The crystal structure of Ara h 2 in complex with D08 Fab fragment**

The Ara h2 + D08 IgE Fab immunocomplex was crystallized at 20° C by hanging drop vapor diffusion method. The protein concentration was 4.35 mg/ml. The reservoir contained 450 µl 10% PEG 2000, 0.2M MgCl<sub>2</sub> in 0.1M Na citrate pH 5 buffer and 50 µl  
10    ethanol as an additive. Crystals were grown using equal volumes (2.5 µl) of the protein solution and the reservoir solution. Small crystals appeared in the drops after three to four months. The grown crystals were soaked in reservoir solution containing 25 % glycerol as a cryoprotectant, mounted in loops and stored in liquid nitrogen. The X-ray diffraction data was collected at MASSIF-1 beamline, ESRF, France. All diffraction data were processed  
15    and scaled. Immunocomplex crystallized in space group P212121 with cell dimensions a=125.0, b=139.5, c=226.6 Å. Data was processed at 3.2 Å resolution which resulted in 126142 unique reflections (99.9 %) with R-factor 48.5 %. The structure was solved by using molecular replacement. The AlphaFold predicted D08 Fab model was used as a search model. The resulted electron density map revealed the binding of three motifs  
20    containing loop for Ara h 2, but the core protein of Ara h 2 was disordered and not possible to trace. The model was corrected manually and refined. The final model contains two immunocomplex structures in the asymmetric unit. In both immunocomplexes three D08 Fab molecules are bound to same long loop of Ara h 2. The final model has an R-factor 22.7 % and a free R-factor 29.5 %.

25    **EXAMPLE 6. Design of the Ara h 2 mutations (A2v1, A2v2, A2v3, A2v4, A2v5, A2v6, A2v7 and A2v8)**

The goal in the hypoallergen design is to achieve a mutant allergen whose ability to bind and cross-link IgE-antibodies on the mast-cell surface is strongly reduced but which still maintains a very similar structure as the wild type allergen. This  
30    would favour the induction of IgG and other antibodies which would have ability to bind both to wild-type allergen and mutant allergen.

The determined immunocomplex structure showed similar binding of DPYSPS motifs in all three D08 Fabs. A closer inspection revealed that the aspartic acid (D) is not interacting with D08 IgE Fab. The remaining five residues (PYSPS) were all mutated with 19 amino acids and ranked according to how favourable/unfavourable interactions with D08 IgE are. Because the binding pocket is hydrophobic, it is reasonable that polar or even charged residue mutations are beneficial to decrease IgE binding. In addition, the side chains which would be too large to fit to binding pocket would be potential ones. Based on this analysis Variants 1-4 with alternative amino acid changes in all three motifs were selected to test experimentally.

The long loop containing three DPYSPS motifs were deleted in Variant 5. On the bases of structural information, we deduced to delete residues R41 to S66. In addition, we introduced the mutation P67A to facilitate correct folding of this variant.

Variant 6 design is based on combination of principles used to design Variant 1 and Variant 5. The first two motifs are deleted, and the third motif contains similar mutations as in Variant 1.

Because Variant 5 (in which all three motifs were deleted) showed more extensive reduction in IgE binding compared to wt Ara h 2 we concluded that the deletion has changed local conformation of protein and reduced binding to conformational epitope of Ara h 2. Therefore, we visually investigated the Ara h 2 structure in the N- and C-terminal regions of three motif containing loop and designed additional Y68D mutation on the putative IgE epitope. In consequence, Variant 7 contains mutations as in Variant 1 + additional putative conformational epitope mutation at Y68D.

Variant 8 is based on Ara h 2.0101 (short) and has similar mutations as used in Variant 1 in both two motifs.

#### EXAMPLE 7. Competitive IgE binding assay

For the competitive IgE binding assay natural Ara h 2 (InBio) containing both isoforms Ara h 2.0101 and Ara h 2.0201 was biotinylated according to the manufacturer's instructions using a 10-fold molar excess of EZ-Link Sulfo-NHS-

LC-Biotin reagent (ThermoFisher Scientific). Free biotin was removed with an EconoPac 10 DG column (Bio-Rad). Biotinylated natural Ara h 2 was spotted as three concentrations (100, 50 and 25 µg/ml, 1 nl/spot) onto the streptavidin coated microtiter plate wells (Pierce™ Streptavidin Coated High Capacity Plates, 5 ThermoFisher Scientific) by a Nano-Plotter 2.1 (GeSiM). Serum samples from peanut allergic adult donors were purchased from PlasmaLab International (Everett WA, USA). Ara h 2 specific IgE had been determined by ImmunoCap™ (ThermoFisher Scientific) by PlasmaLab. The optimal dilution for each serum sample was analyzed by an IgE serum titration analysis for each spotted 10 concentration of the natural Ara h 2 (data not shown). For the competitive IgE binding assay, diluted sera was pre-incubated with different amounts of competing allergen in final allergen concentrations of 10 000, 2 000, 400, 80, 16, 3, 0.6 and 0 pM and then added to the well in which the biotinylated natural Ara h 2 was immobilized. All IgE binding experiments were performed in duplicates. 15 Peroxidase-conjugated anti-human IgE (Southern Biotech) was used for detection and fluorescently-labelled tyramide (ThermoFisher Scientific) for signal amplification. The fluorescence readout was obtained by scanning the wells with LS400 microarray scanner and 633 nm laser (Tecan). Image analysis, spot detection and fluorescence intensity quantification were performed with Array-Pro 20 Analyzer software (Media Cybernetics). Local background signal value was measured outside the specific spots from each well. The net signal value for each spot was obtained by subtracting the local background signal value from the average intensity value of each spot.

The IgE binding of a serum pool prepared from nine individual samples were first 25 analyzed by the competitive immunoassay (Fig. 2). Natural Ara h 2 was inhibiting IgE binding of the serum pool whereas variants A2v1-A2v5 showed significantly weaker inhibition capacity.

With the individual serum samples the variants A2v1 and A2v5 show significantly reduced inhibition when compared to the natural Ara h 2 control indicating that the designed 30 mutations of Ara h 2 variants locate in an important IgE epitope area (Fig.3).

#### EXAMPLE 8. Histamine release assay

Biological activity of natural Ara h 2 and variants A2v1 and A2v5 was analyzed by a

histamine release assay (HRA). HRA was performed as an outsourced service at RefLab Aps (Copenhagen, Denmark). Briefly, stripped human basophils were passively sensitized with Ara h 2 proteins in 16 different concentrations (from 0.01 to 10 ng/ml) using three serum samples. Released histamine induced by different allergen concentrations was measured as duplicates by ELISA (enzyme-linked immunosorbent assay) using a glass microfibre method developed by RefLab (Skov et al. 1985). The results of the HRA show that the biological activity of A2v1 and A2v5 is significantly lower compared to the natural Ara h 2 with all three serum samples (Fig.4).

EXAMPLE 9. Mass spectrum measurements for modified Ara h 2 variants

Mass-spectrometric experiments were performed as in Example 2.

**Table 2.** High resolution ESI FT-ICR mass spectrum analysis of the recombinant Ara h 2 allergens

| Recombinant Ara h 2 allergen | Observed mass (Da) | Theoretical mass (Da) | Mass difference (Da) | SEQ ID |
|------------------------------|--------------------|-----------------------|----------------------|--------|
| rAra h 2.0101                | 16713.71           | 16713.70              | + 0.01               | NO:3   |
| rAra h 2.0201                | 18056.31           | 18056.30              | + 0.01               | NO:2   |
| rAra h 2 variant 1           | 18007.18           | 18007.16              | + 0.02               | NO:4   |
| rAra h 2 variant 2           | 18049.26           | 18049.21              | + 0.05               | NO:5   |
| rAra h 2 variant 3           | 18004.37           | 18004.32              | + 0.05               | NO:6   |
| rAra h 2 variant 4           | 18058.26           | 18058.24              | + 0.02               | NO:7   |
| rAra h 2 variant 5           | 15034.96           | 15037.98              | - 3.02               | NO:8   |
| rAra h 2 variant 6           | 16689.78           | 16689.71              | + 0.07               | NO:9   |
| rAra h 2 variant 7           | 17959.17           | 17959.13              | + 0.04               | NO:10  |
| rAra h 2 variant 8           | 16681.67           | 16681.60              | + 0.07               | NO:11  |

Based on the determined molecular weights of the recombinant Ara h 2 allergen variants 1-8 shown in Table 2, the four disulphide bridges of Ara h 2 required for the correct folding are formed in all recombinant Ara h 2 allergens except for the Ara h 2 variant 5 showing a minus 3 Dalton difference between the observed and theoretical mass. The whole loop region containing the three DPYSPS peptide motifs has been deleted in the Ara h 2 variant 5 which might lead to problems in the correct folding of the Ara h 2 variant 5 and thus to

heterogeneity of the purified protein. In the Ara h 2 variant 6, two of the DPYSPS peptide motifs have been deleted and the third motif has mutations in the third (Y/E) and fifth (P/D) amino acid.

**EXAMPLE 10. Competitive IgE binding assay for variants 4, 6 and 8.**

- 5 Experiments were done as in Example 7 with the exception of the concentrations of competing soluble allergens. Competition with natural Ara h 2 was carried out as in Example 7 with final concentrations of 10 000, 2 000, 400, 80, 16, 3, 0.6 and 0 pM. Competition with Ara h 2 variants 4, 6 and 8 were carried out with the final concentrations of 40 000, 8 000, 1 600, 320, 64, 12, 2.4 and 0 pM.
- 10 The IgE binding of a serum pool prepared from nine individual samples was first analyzed by the competitive immunoassay (Fig. 5). Natural Ara h 2 inhibit strongly IgE binding of the serum pool whereas variants A2v4, A2v6 and A2v8 show significantly weaker inhibition capacity. With the individual serum samples the variants A2v4, A2v6 and A2v8 show significantly reduced inhibition when compared to the natural Ara h 2 control
- 15 indicating that the designed 3D mutations of Ara h 2 variants 4, 6 and 8 locate in an important IgE epitope area (Fig. 6).

These results show that correctly folded Ara h 2 variant 6 having similar decrease in the IgE binding (Figs. 5 and 6) and histamine release (Fig. 7) compared to Ara h 2 variant 5 should provide an efficient protective IgG response during immunotherapy.

20 **EXAMPLE 11. Histamine release assay**

- Biological activity of natural Ara h 2 and variants A2v4 and A2v6 was analyzed by a histamine release assay (HRA). HRA was performed as an outsourced service at RefLab Aps (Copenhagen, Denmark). Briefly, stripped human basophils were passively sensitized with Ara h 2 proteins in 16 different concentrations (from 0.01 to 10 ng/ml) using two
- 25 serum samples. Released histamine induced by different allergen concentrations was measured as duplicates by ELISA (enzyme-linked immunosorbent assay) 5 using a glass microfibre method developed by RefLab (Skov et al. 1985). The results of the HRA show that the biological activity of A2v4 and A2v6 is significantly lower compared to the natural Ara h 2 with both serum samples (Fig. 7).

**EXAMPLE 12. Preparation of Ara h 2 variants in *E.coli*.**

The productivity of Ara h 2 variants was tested by expressing Ara h 2 variant 6 of the present disclosure and Ara h 2 variant B1001 described in WO 2023/012652 A2 in the BL21 *E.coli* strain using the same expression vector pET28b+ under otherwise identical  
5 conditions, as described in Example 1. The insoluble protein fraction isolated from the cell pellets was solubilized and analyzed by Coomassie stained SDS-PAGE and the intensity of the Ara h 2 variant 6 was scanned and visually inspected. The expression level of Ara h 2 variant 6 (see Fig. 8, lane 3) was comparable to other Ara h 2 variants described in Example 1 but the expression level of Ara h 2 variant B1001 in this setting was so low that  
10 the expressed product was hardly visible in the gel (see Fig. 8, lanes 5, 6, 7, and 8).

**CITATION LIST****Patent Literature**

US20090297564

US20100166802

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WO2008092992

WO2023012652

WO2023060151

**Non-Patent Literature**

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## CLAIMS:

1. A modified Ara h 2 peanut allergen polypeptide, wherein the wild type DPYSPS amino acid motifs of SEQ ID NO:1 in the polypeptide, the proline (P) at position 5 of SEQ ID  
5 NO:1 of the wild type motif being a hydroxyproline, comprise a first amino acid substitution at position 3 of SEQ ID NO:1, and a second amino acid substitution at position 5 of SEQ ID NO:1, wherein said modified Ara h 2 peanut allergen polypeptide is a mutant of peanut allergen polypeptide Ara h 2.0201 of SEQ ID NO:2 comprising three DPYSPS amino acid motifs, or of peanut allergen polypeptide Ara h 2.0101 of SEQ ID NO:3  
10 comprising two DPYSPS amino acid motifs, and wherein the amino acid sequence of said modified polypeptide has at least 90% sequence identity with SEQ ID NO:2 or 3 outside said DPYSPS motifs, and wherein positions 1, 4, and 6 of SEQ ID NO:1 are not modified in said DPYSPS motif(s) of said modified Ara h 2 peanut allergen polypeptide.
2. The modified Ara h 2 peanut allergen polypeptide according to any one of claims 1-3,  
15 wherein at least one DPYSPS motif has been deleted in said modified Ara h 2 peanut allergen polypeptide so that said polypeptide comprises only one or two DPYSPS motif(s), wherein said remaining one or two DPYSPS motif(s) is/are modified as defined in claim 1.
3. The modified Ara h 2 peanut allergen polypeptide according to claim 2, wherein said modified Ara h 2 peanut allergen polypeptide comprises only two remaining DPYSPS  
20 motifs, wherein said two DPYSPS motifs are modified as defined in claim 1.
4. The modified Ara h 2 peanut allergen polypeptide according to claim 2, wherein said modified Ara h 2 peanut allergen polypeptide comprises only one remaining DPYSPS motif, wherein said DPYSPS motif is modified as defined in claim 1.
5. The modified Ara h 2 peanut allergen polypeptide according to claim 1, wherein said  
25 first amino acid substitution at position 3 of SEQ ID NO:1 is from tyrosine (Y) to an amino acid residue selected from the group consisting of: glutamic acid (E), lysine (K), tryptophan (W), aspartic acid (D), asparagine (N), arginine (R), cysteine (C), glutamine (Q), serine (S), threonine (T), alanine (A), isoleucine (I), glycine (G), and proline (P).
6. The modified Ara h 2 peanut allergen polypeptide according to claim 5, wherein said  
30 first amino acid substitution at position 3 of SEQ ID NO:1 is from tyrosine (Y) to glutamic acid (E) or lysine (K).

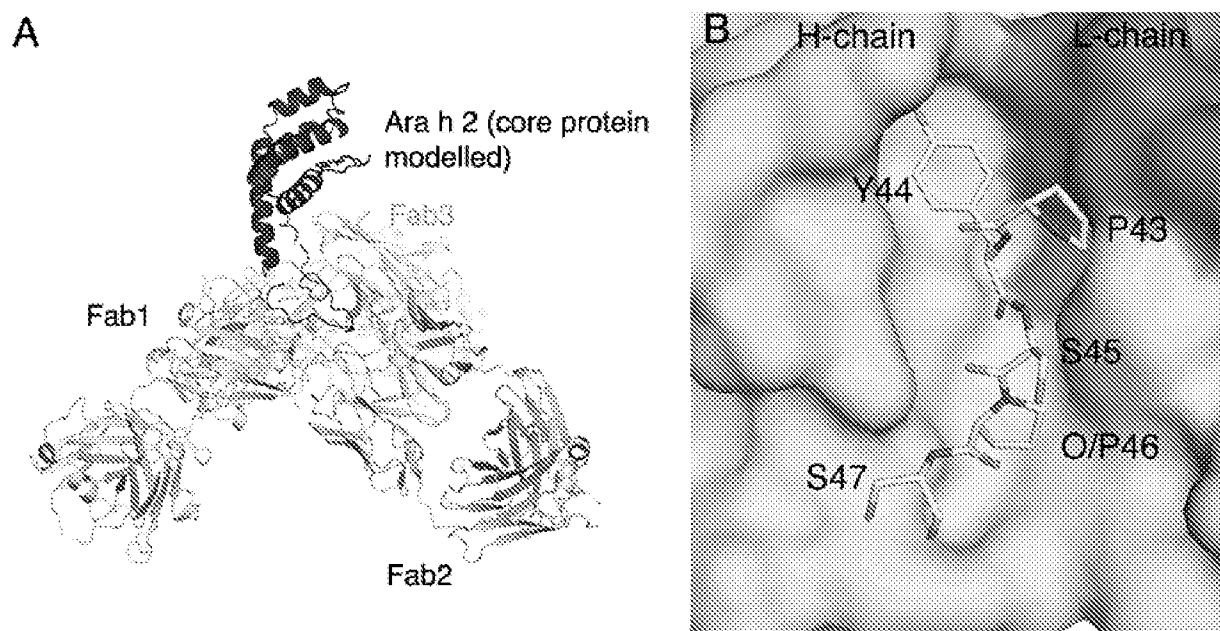
7. The modified Ara h 2 peanut allergen polypeptide according to claim 1, wherein said second amino acid substitution at position 5 of SEQ ID NO:1 is from proline (P) to an amino acid residue selected from the group consisting of: aspartic acid (D), glutamic acid (E), tyrosine (Y), tryptophan (W), phenylalanine (F), leucine (L), isoleucine (I),  
5 methionine (M), lysine (K), arginine (R), and histidine (H).
8. The modified Ara h 2 peanut allergen polypeptide according to claim 7, wherein said second amino acid substitution at position 5 of SEQ ID NO:1 is from proline (P) to aspartic acid (D) or glutamic acid (E).
9. The modified Ara h 2 peanut allergen polypeptide according to any one of claims 5-8,  
10 wherein each of said two or three DPYSPS amino acid motifs in said modified Ara h 2 peanut allergen polypeptides comprises a third amino acid substitution at position 2 of SEQ ID NO:1.
10. The modified Ara h 2 peanut allergen polypeptide according to claim 9, wherein said third amino acid substitution at position 2 of SEQ ID NO:1 is from proline (P) to an amino  
15 acid residue selected from the group consisting of: aspartic acid (D), glutamic acid (E), tyrosine (Y), tryptophan (W), phenylalanine (F), leucine (L), and isoleucine (I).
11. The modified Ara h 2 peanut allergen polypeptide according to claim 9, wherein said third amino acid substitution at position 2 of SEQ ID NO:1 is from proline (P) to aspartic acid (D).
- 20 12. The modified Ara h 2 peanut allergen polypeptide according to claim 1, wherein said amino acid modifications are amino acid substitutions at positions defined by the Ara h 2.0201 wild type sequence of SEQ ID NO:2 and said positions are selected from the group consisting of positions: P43, Y44, P46, P50, Y51, P53, Y63, and P65, wherein said modified Ara h 2 peanut allergen polypeptide has an amino acid sequence having at least  
25 90% sequence identity with the amino acid sequence of SEQ ID NO:2 outside the DPYSPS motifs.
13. The modified Ara h 2 peanut allergen polypeptide according to claim 12, wherein said amino acid modifications are selected from the group consisting of: P43D, Y44E, Y44K, P46D, P46E, P50D, Y51E, Y51K, P53D, P53E, P62D, Y63E, Y63K, P65D, and P65E.

14. The modified Ara h 2 peanut allergen polypeptide according to claim 13, wherein said amino acid modifications are Y44E, P46D, Y51E, P53D, Y63E, and P65D.
15. The modified Ara h 2 peanut allergen polypeptide according to claim 13, wherein said amino acid modifications are Y44E, P46E, Y51E, P53E, Y63E, and P65E.
- 5 16. The modified Ara h 2 peanut allergen polypeptide according to claim 13, wherein said amino acid modifications are Y44K, P46D, Y51K, P53D, Y63K, and P65D.
17. The modified Ara h 2 peanut allergen polypeptide according to claim 13, wherein said amino acid modifications are P43D, Y44K, P46D, P50D, Y51K, P53D, P62D, Y63K, and P65D.
- 10 18. The modified Ara h 2 peanut allergen polypeptide according to claim 2, wherein at least one DPYSPS (SEQ ID NO:1) amino acid motif in the polypeptide has been deleted, wherein said modified Ara h 2 peanut allergen polypeptide is a mutant of peanut allergen polypeptide Ara h 2.0201 of SEQ ID NO:2 comprising three DPYSPS amino acid motifs, or of peanut allergen polypeptide Ara h 2.0101 of SEQ ID NO:3 comprising two DPYSPS  
15 amino acid motifs, and wherein remaining DPYSPS (SEQ ID NO:1) amino acid motif(s) has/have been modified so that the motif comprises a first amino acid substitution at position 3 of SEQ ID NO:1, and a second amino acid substitution at position 5 of SEQ ID NO:1.
19. The modified Ara h 2 peanut allergen polypeptide according to claim 18, wherein the  
20 amino acid at position P67 defined by the Ara h 2.0201 wild type sequence of SEQ ID NO:2 comprises an amino acid substitution, preferably said amino acid modification is P67A, and/or the amino acid at position Y68 defined by the Ara h 2.0201 wild type sequence of SEQ ID NO:2 comprises an amino acid substitution, preferably said amino acid modification is Y68D.
- 25 20. A modified Ara h 2 peanut allergen polypeptide, wherein all DPYSPS (SEQ ID NO:1) amino acid motifs have been deleted, wherein said modified Ara h 2 peanut allergen polypeptide is a mutant of peanut allergen polypeptide Ara h 2.0201 of SEQ ID NO:2 comprising three DPYSPS amino acid motifs, or of peanut allergen polypeptide Ara h 2.0101 of SEQ ID NO:3 comprising two DPYSPS amino acid motifs.

21. The modified Ara h 2 peanut allergen polypeptide according to claim 20, wherein said deleted area in the polypeptide corresponds to positions R41-S66 of the Ara h 2.0201 wild type sequence of SEQ ID NO:2 or to positions R41-S54 of the Ara h 2.0101 of SEQ ID NO:3.
- 5 22. The modified Ara h 2 peanut allergen polypeptide according to claim 20 or 21, wherein said modified Ara h 2 peanut allergen polypeptide has an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:2 outside the DPYSPS motifs at positions between R41-S66 and P67 of SEQ ID NO:2, or at least 90% sequence identity to the amino acid sequence of SEQ ID NO:3 outside the DPYSPS motifs  
10 at positions between R41-S54 of SEQ ID NO:3.
23. The modified Ara h 2 peanut allergen polypeptide according to any one of claims 20-22, wherein the amino acid at position(s) P67 and/or Y68 defined by the Ara h 2.0201 wild type sequence of SEQ ID NO:2 comprise(s) an amino acid substitution, preferably said amino acid modification is P67A or Y68D.
- 15 24. The modified Ara h 2 peanut allergen polypeptide according to any one of claims 1-23, wherein the modified Ara h 2 peanut allergen polypeptide activates release of histamine from basophils to a degree less than a corresponding wild type allergen.
25. A pharmaceutical composition comprising the modified Ara h 2 peanut allergen polypeptide according to any of claims 1-24 and at least one of the following:  
20 physiologically acceptable adjuvant, carrier, diluent, excipient, preservative and stabilizer.
26. The pharmaceutical composition according to claim 25, wherein said composition is a vaccine composition comprising a pharmaceutically acceptable diluent or adjuvant.
27. A method of treating peanut allergy in a subject, the method comprising: administering to the subject an amount of the modified Ara h 2 peanut allergen polypeptide according to  
25 any one of claims 1-24 in an amount effective to ameliorate at least one symptom or clinical sign of allergy to the peanut Ara h 2 allergen, wherein the modified Ara h 2 peanut allergen polypeptide activates release of histamine from basophils to a degree less than the wild type allergen.
28. A method of producing a modified Ara h 2 peanut allergen polypeptide for  
30 immunotherapy comprising the steps of:

- a) modifying nucleic acid sequence encoding a wild type Ara h 2 peanut allergen polypeptide in order to substitute at least one of the wild type DPYSPS amino acid motifs of SEQ ID NO:1 in the polypeptide with a modified DPYSPS motif comprising a first amino acid substitution at position 3 of SEQ ID NO:1, and a second amino acid substitution at position 5 of SEQ ID NO:1, and wherein positions 1, 4, and 6 of SEQ ID NO:1 are not modified in said modified DPYSPS motif, wherein said modified Ara h 2 peanut allergen polypeptide is preferably a mutant of peanut allergen polypeptide Ara h 2.0201 of SEQ ID NO:2 comprising three DPYSPS amino acid motifs, or of peanut allergen polypeptide Ara h 2.0101 of SEQ ID NO:3 comprising two DPYSPS amino acid motifs;
- b) expressing or producing the modified Ara h 2 peanut allergen polypeptide from the modified nucleic acid; and
- c) isolating and purifying the modified Ara h 2 peanut allergen polypeptide from step b).
29. The method according to claim 28 comprising a further step of: d) testing the modified Ara h 2 peanut allergen polypeptide obtained from step b) or c) for ability to activate release of histamine of an allergic reactions from human cells, wherein those the modified Ara h 2 peanut allergen polypeptides which do not activate histamine release or which activate histamine release less than the corresponding wild type Ara h 2 polypeptide are considered as hypoallergen candidates for immunotherapy.
30. The method according to claim 28 or 29 further comprising a step of: e) analysing that said modified Ara h 2 peanut allergen polypeptide has a native-like structure and immunogenic potential to develop protective IgG antibodies.
31. The method according to any one of claims 28-30, wherein the nucleic acid sequence encoding a wild type Ara h 2 polypeptide is modified so that the amino acid substitutions formed are at positions defined by the Ara h 2.0201 wild type sequence of SEQ ID NO:2 and said substitutions are selected from the group consisting of: P43D, Y44E, Y44K, P46D, P46E, P50D, Y51E, Y51K, P53D, P53E, P62D, Y63E, Y63K, P65D, and P65E.
32. The method according to claim 31, wherein said amino acid substitutions are i) Y44E, P46D, Y51E, P53D, Y63E, and P65D; ii) Y44E, P46E, Y51E, P53E, Y63E, and P65E; iii) Y44K, P46D, Y51K, P53D, Y63K, and P65D; or iv) P43D, Y44K, P46D, P50D, Y51K, P53D, P62D, Y63K, and P65D.

33. An isolated nucleic acid sequence encoding the modified Ara h 2 peanut allergen polypeptide according to any one of claims 1-24.
34. An isolated vector comprising a nucleic acid sequence encoding the modified Ara h 2 peanut allergen polypeptide according to any one of claims 1-24.
- 5 35. An isolated host cell comprising a vector comprising a nucleic acid sequence encoding the modified Ara h 2 peanut allergen polypeptide according to any one of claims 1-24.



**Figures 1A and 1B.**

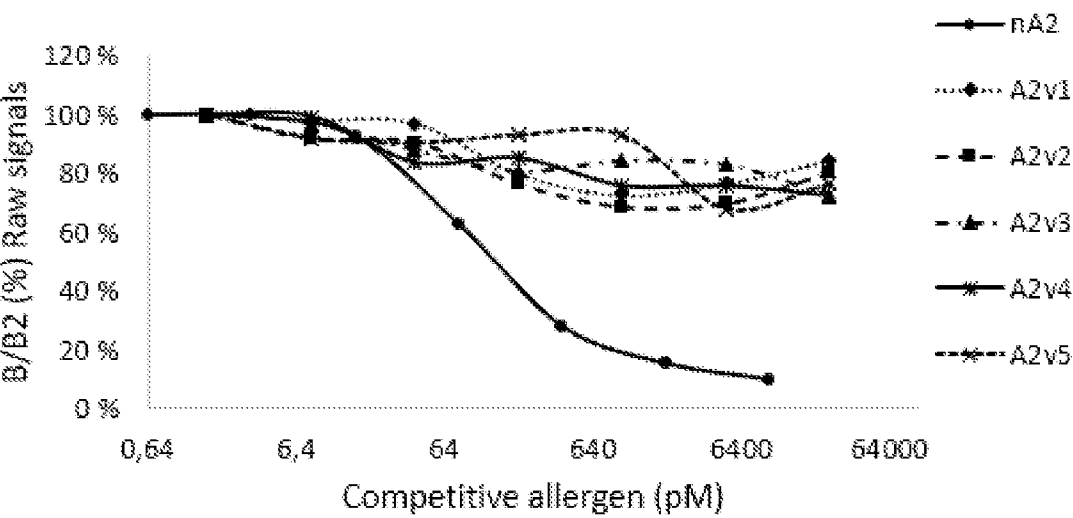
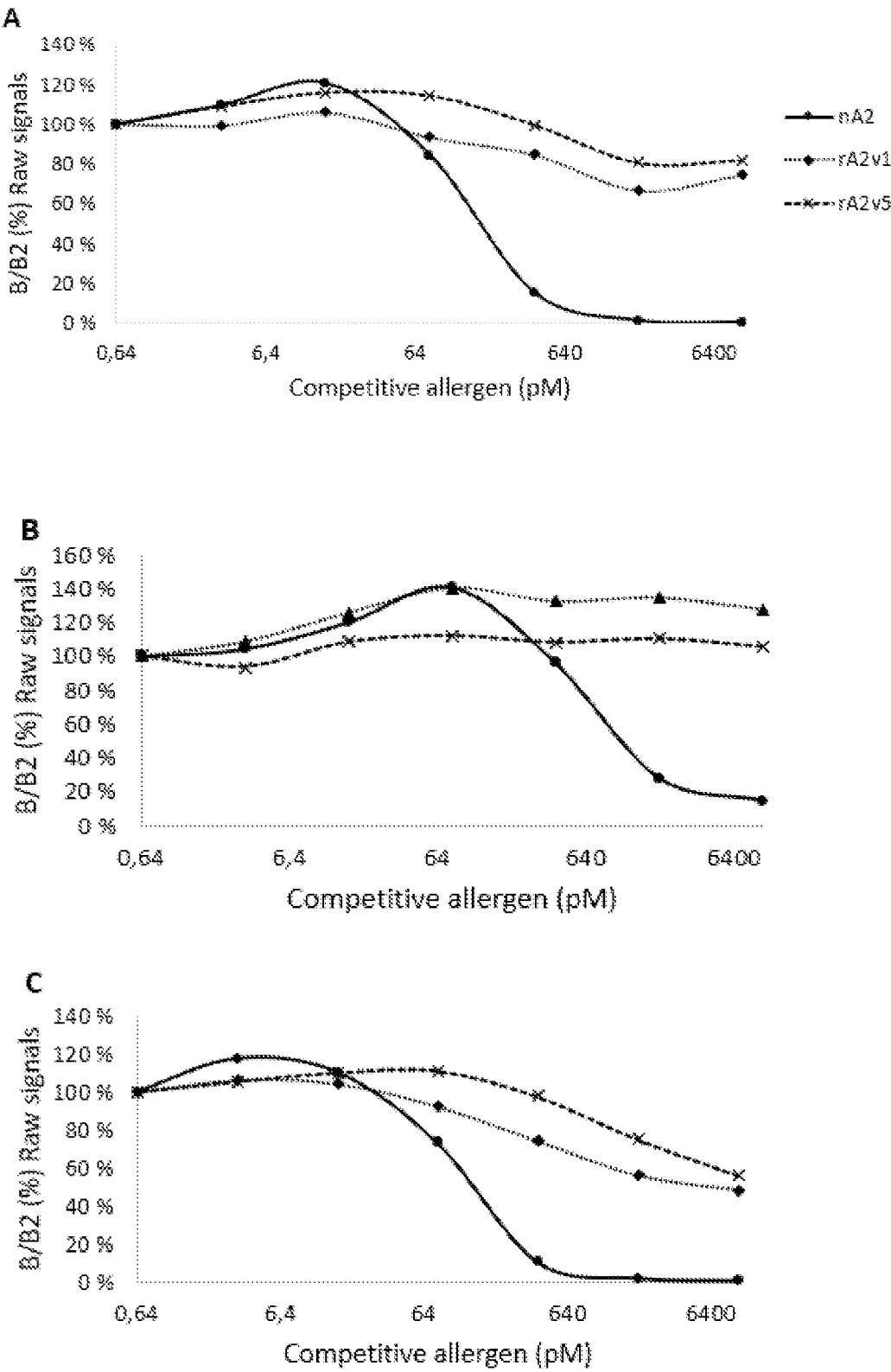
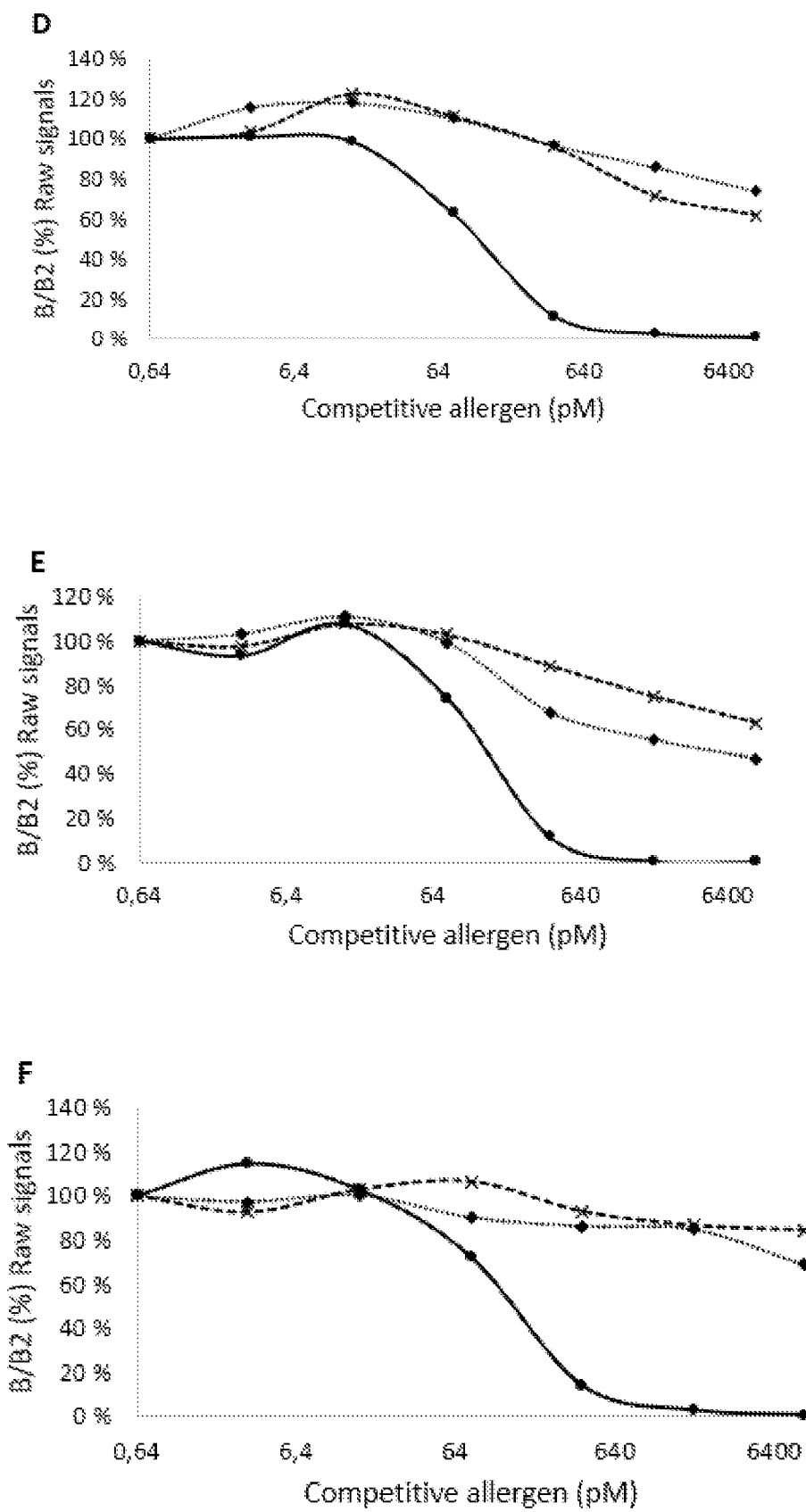


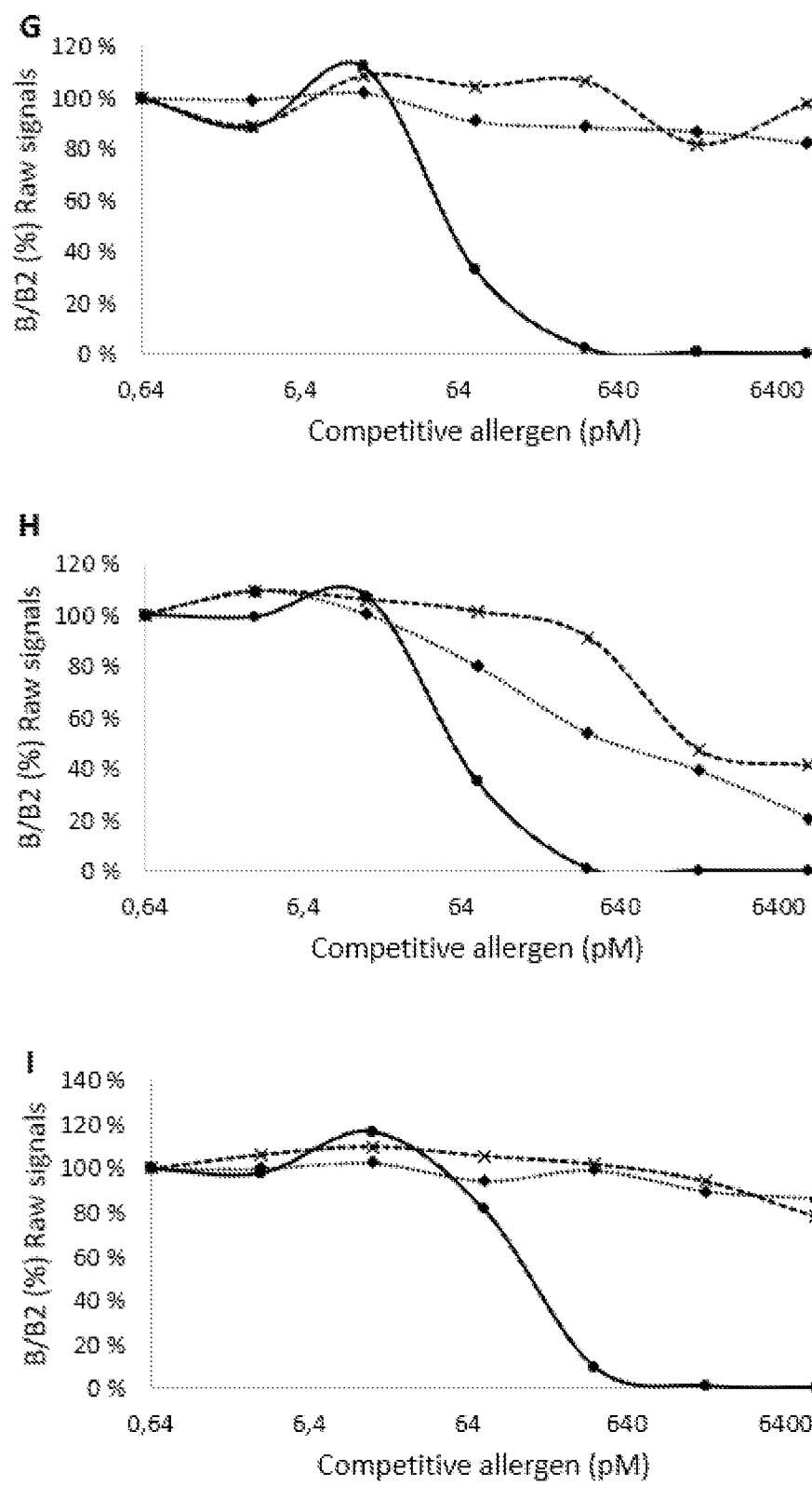
Figure 2.



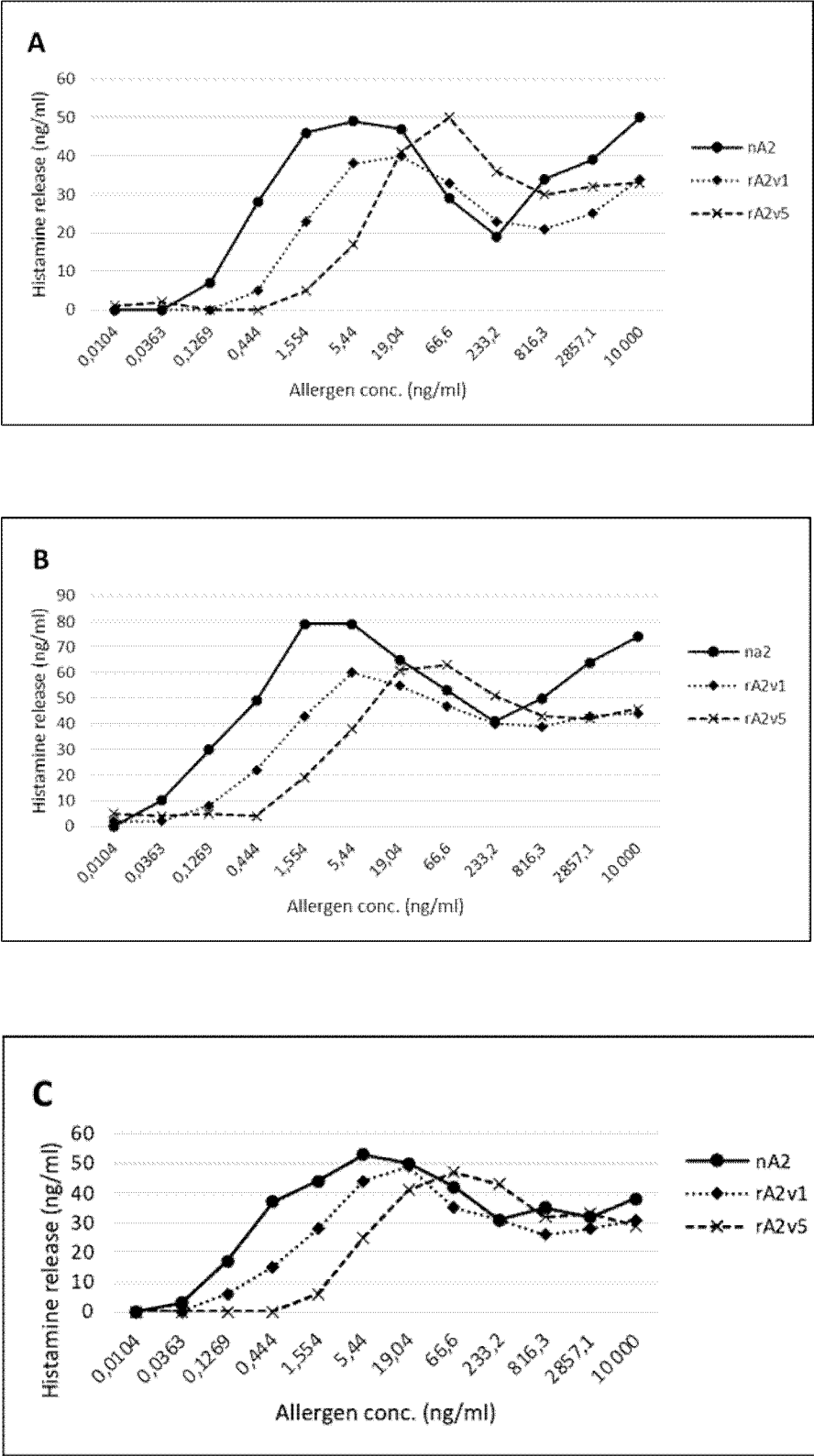
Figures 3A-3C.



Figures 3D-3F.



Figures 3G-3I.



Figures 4A-4C.

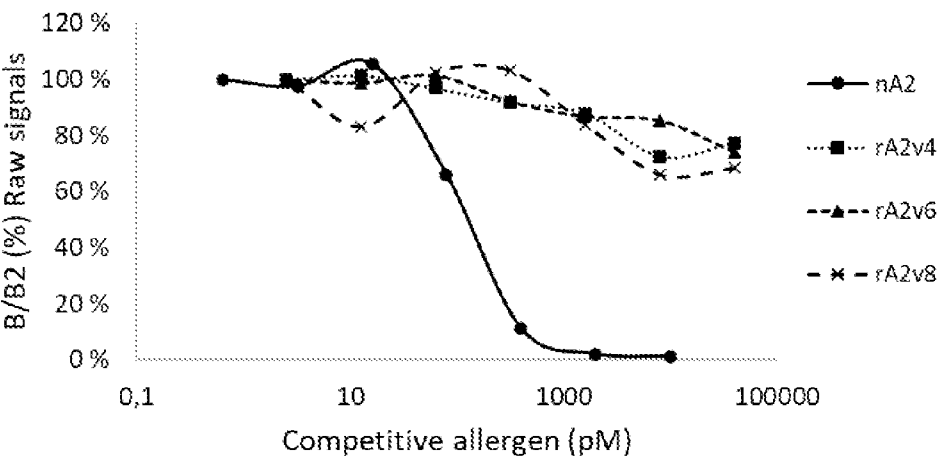


Figure 5.

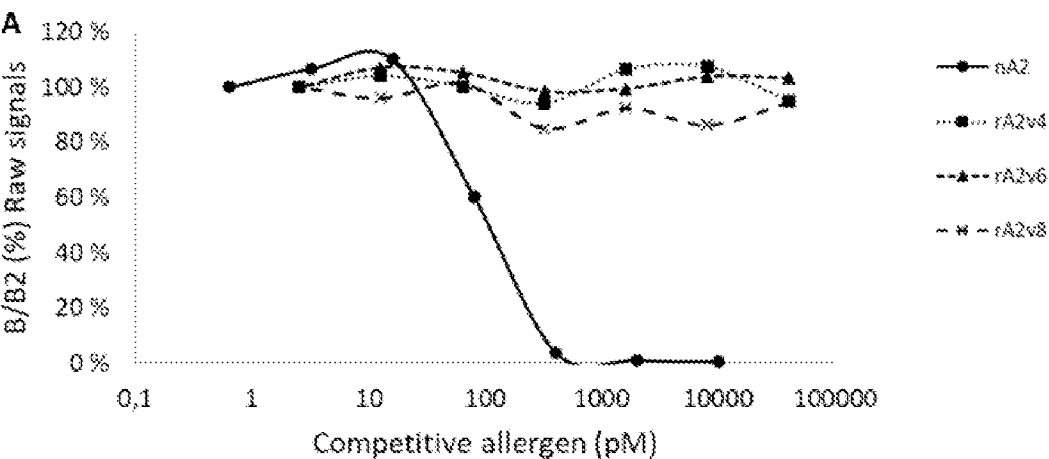
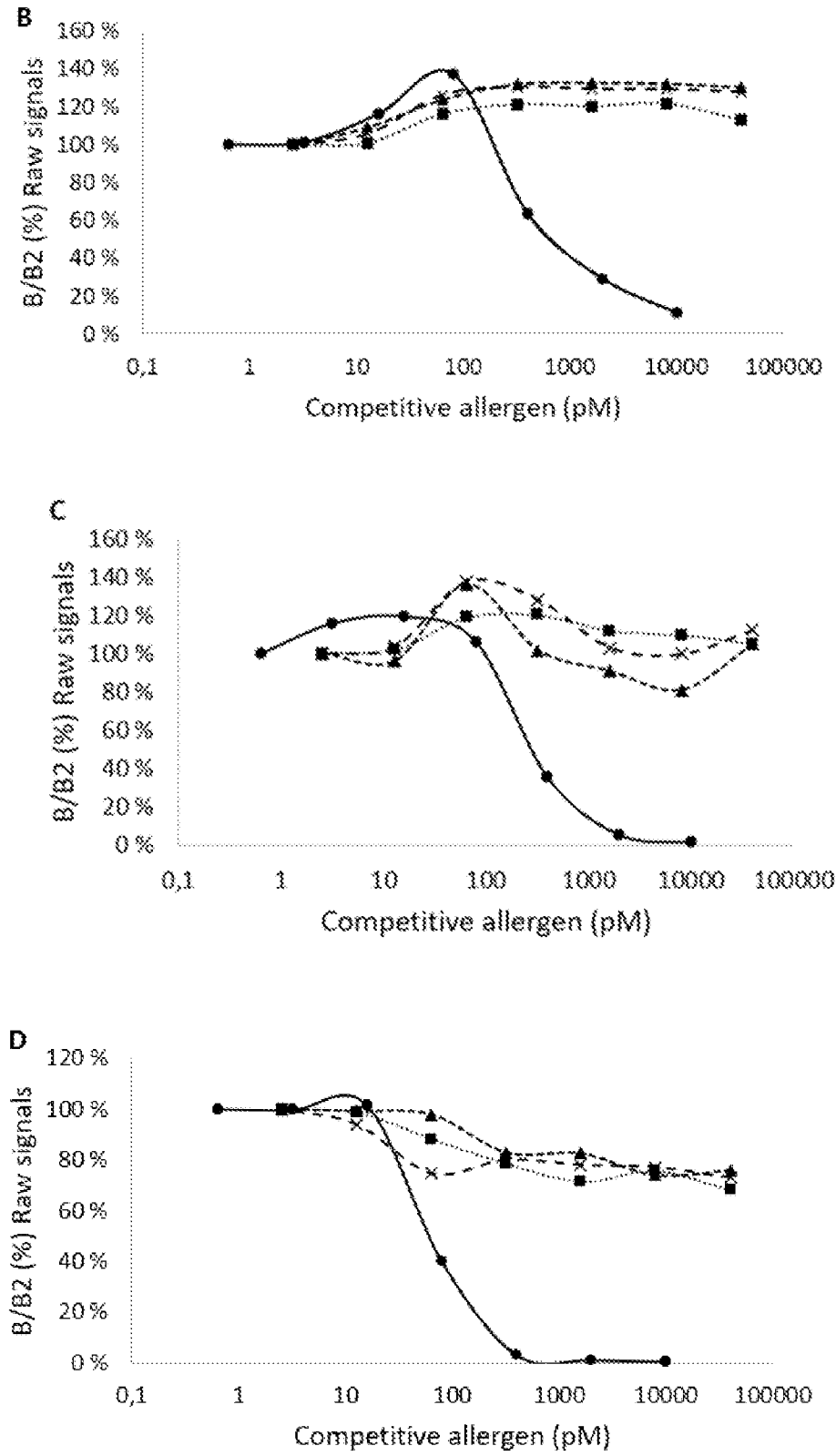
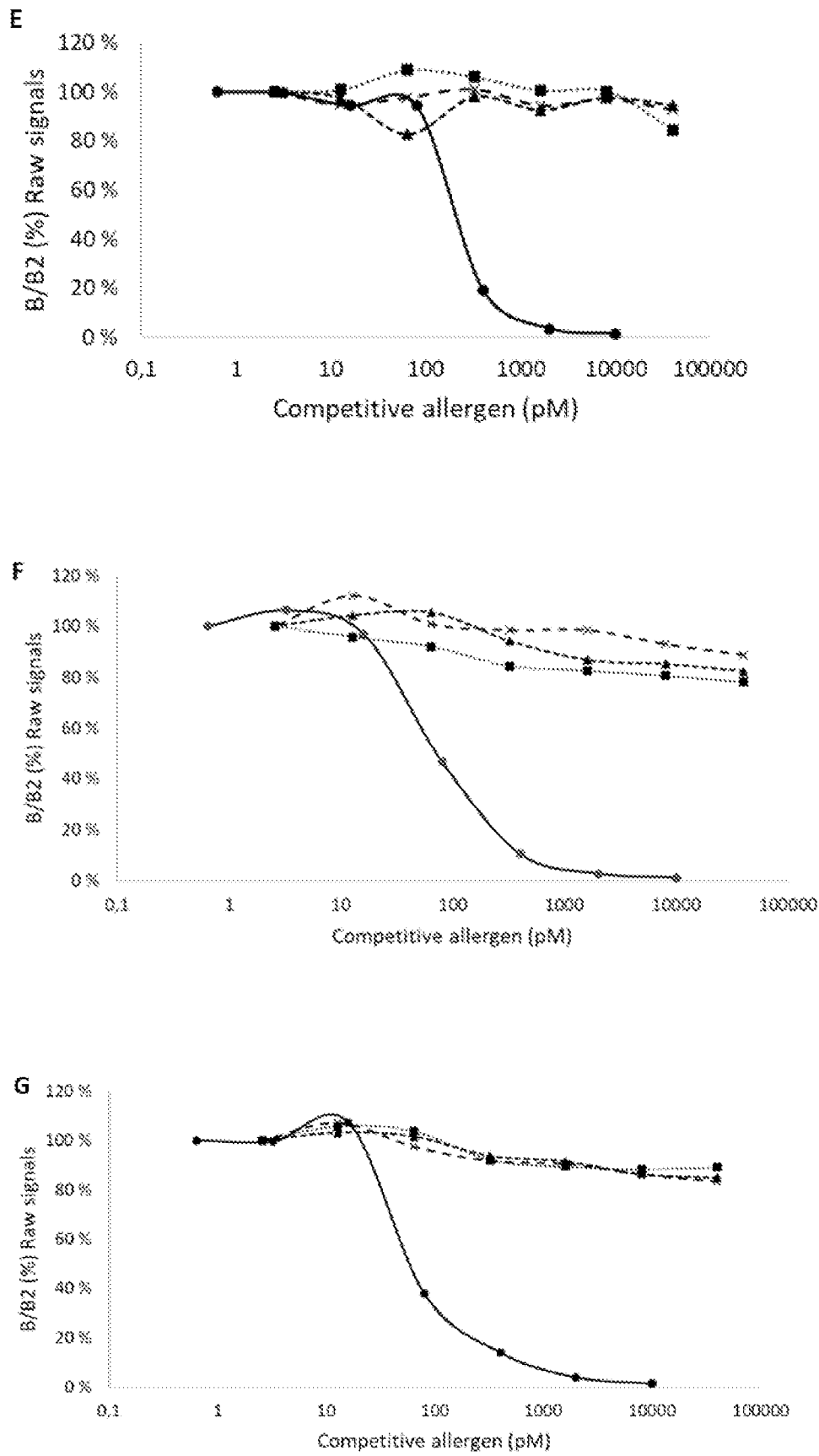


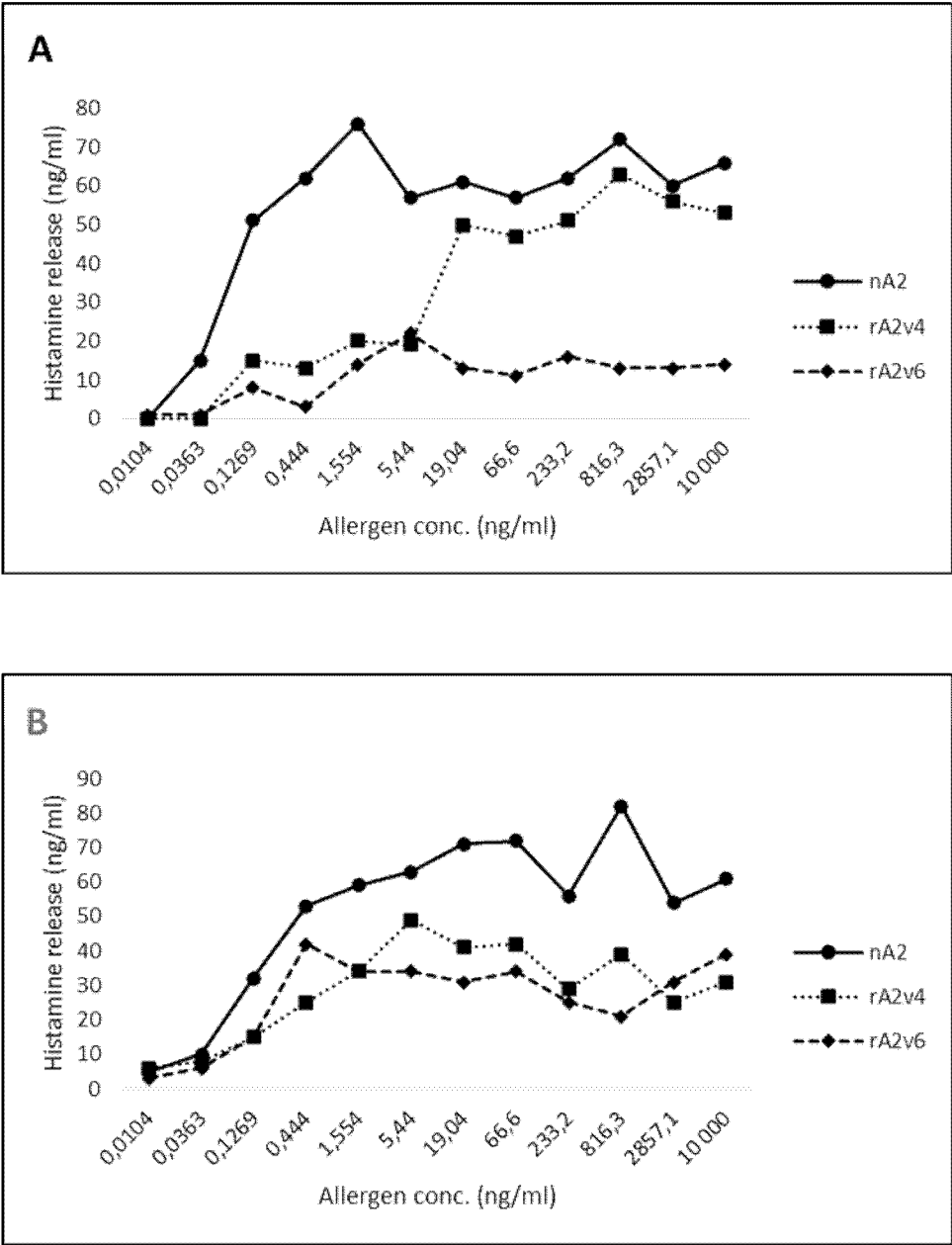
Figure 6A.



Figures 6B-6D.



Figures 6E-6G.



Figures 7A and 7B.

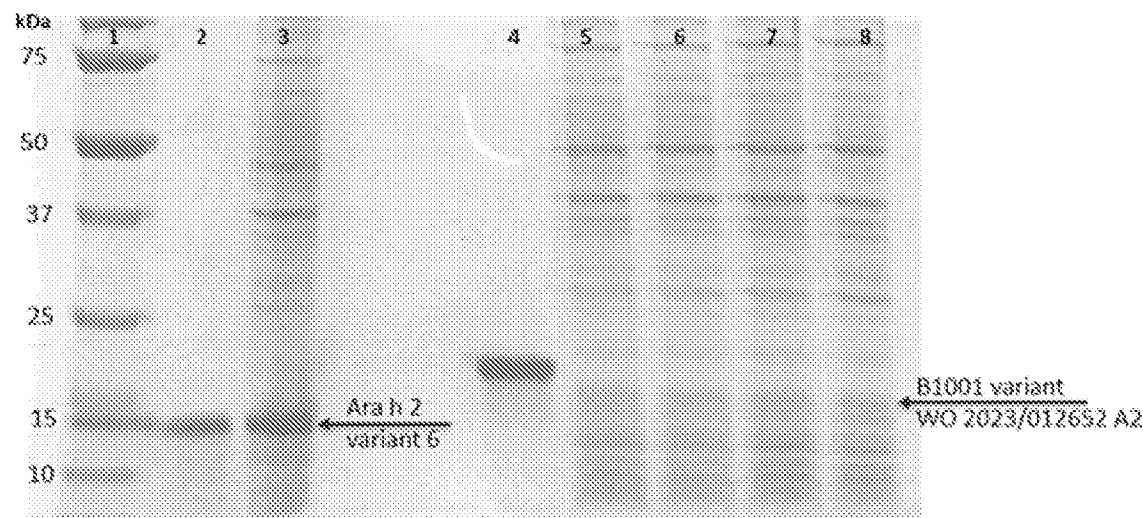


Figure 8.

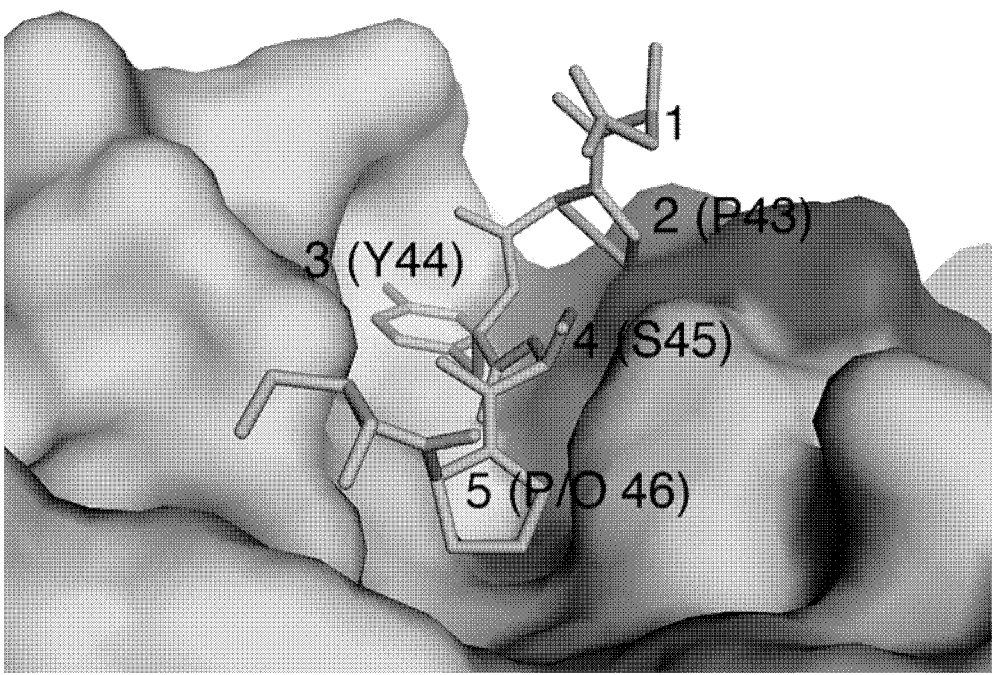


Figure 9.

## INTERNATIONAL SEARCH REPORT

International application No

PCT/FI2024/050299

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. C07K14/415 A61K39/35

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                        | Relevant to claim No. |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | WO 2023/012652 A2 (UKKO INC [US])<br>9 February 2023 (2023-02-09)<br>abstract<br>paragraph [0010] - paragraph [0011]<br>example 2; table 2<br>example 4; tables 3, 4<br>claims 1-6, 9, 11, 12, 13, 15<br>-----<br>- / - - | 1 - 19 ,<br>24 - 35   |



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

9 August 2024

Date of mailing of the international search report

21/10/2024

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2

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Authorized officer

Mundel, Christophe

## INTERNATIONAL SEARCH REPORT

International application No

PCT/FI2024/050299

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                                                                      |                        |
|------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                                   | Relevant to claim No.  |
| A                                                    | <p>BERNARD HERVÉ ET AL: "Allergenicity of peanut component Ara h 2: Contribution of conformational versus linear hydroxyproline-containing epitopes", JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 135, no. 5, 4 December 2014 (2014-12-04), page 1267, XP029574873, ISSN: 0091-6749, DOI: 10.1016/J.JACI.2014.10.025 abstract page 1268, left-hand column, line 15 - line 18 figure 1</p> <p>-----</p> | <p>1-19,<br/>24-35</p> |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI2024/050299

### Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
  - a. ☒ forming part of the international application as filed.
  - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/FI2024/050299

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:  
1-19, 28-32 (completely); 24-27, 33-35 (partially)

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-19, 28-32(completely); 24-27, 33-35(partially)

A modified Ara h 2 peanut allergen polypeptide wherein the DPYSPS motifs of SEQ ID NO: 1 in the polypeptide comprise a first substitution at position 3 of SEQ ID NO: 1 and a second substitution at position 5 of SEQ ID NO: 1, the modified polypeptide having at least 90% sequence identity to SEQ ID NO: 2 or 3 outside the DPYSPS motifs and wherein positions 1, 4 and 6 of SEQ ID NO: 1 are not modified. A pharmaceutical composition comprising the modified Ara h 2 peanut allergen polypeptide. A method of treating peanut allergy in a subject comprising administering said modified Ara h 2 peanut allergen polypeptide. A method of producing such a modified Ara h 2 peanut allergen polypeptide. An isolated nucleic acid encoding the modified Ara h 2 peanut allergen polypeptide. An isolated vector comprising said nucleic acid. An isolated host cell comprising the vector.

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2. claims: 20-23(completely); 24-27, 33-35(partially)

A modified Ara h 2 peanut allergen polypeptide wherein all the DPYSPS amino acid motifs have been deleted. A pharmaceutical composition comprising said Ara h 2 peanut allergen. A method for treating peanut allergy in a subject comprising administering said Ara h 2 peanut allergen polypeptide. An isolated nucleic acid encoding the modified Ara h 2 peanut allergen polypeptide. An isolated vector comprising said nucleic acid. An isolated host cell comprising said vector.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No  
PCT/FI2024/050299

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
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