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Mapping and conformational analysis of IgE-binding epitopic regions on the molecular surface of the major Ara h 3 legumin allergen of peanut (*Arachis hypogaea*)

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ABSTRACT

Eight distinct sequential IgE-binding epitopes were identified along the amino acid sequence of Ara h 3 using the Spot technology. They essentially correspond to preferentially electropositive regions exposed on the molecular surface of the protein. A few IgE-binding epitopes are coalescent to create more extended IgE-binding regions exposed on the surface of the allergen. Ara h 3 contains a core region corresponding to the cupin motifs and predicted to be preserved upon the trypsin and chymotrypsin attack in the gastro-intestinal tract. Some of the identified IgE-binding epitopes should remain unaltered in the core region to subsequently interact with the local immune system. They most probably account for the strong allergenic potency of Ara h 3. Most of the identified IgE-binding epitopes of Ara h 3 readily differ from the corresponding regions of other legume and tree-nut legumin allergens except for epitope #1 and #7 which are rather conserved essentially in other allergens. These structurally related epitopes could account for some cross-reactions occurring between Ara h 3 and other legumin allergens.

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1. Introduction

Nowadays, peanut allergy has developed in industrial countries to become one among the most potentially severe and life-threatening food allergies, especially in children (Sicherer et al., 2003; Sicherer and Sampson, 2007). Different peanut allergens responsible for both the sensitization and the IgE-binding reactivity of allergic patients have been identified as seed storage proteins. Essentially, three seed storage proteins corresponding to a vicilin (Ara h 1) (Burks et al., 1995), a 2S albumin (Ara h 2) (Viquez et al., 2001) and a legumin (Ara h 3) (Rabjohn et al., 1999; Restani et al., 2005), respectively, have been identified as the major peanut allergens. Other minor allergens have been described which less frequently trigger the synthesis of appreciable amounts of specific IgE in sensitized individuals (de Leon et al., 2007). In the late 1990s,

continuous IgE-binding epitopes have been characterized on Ara h 1 (Burks et al., 1997; Shin et al., 1998), Ara h 2 (Stanley et al., 1997) and Ara h 3 (Rabjohn et al., 1999) by the Bannon's group, using the Spot technique performed with 15-mer synthetic peptides overlapping by eight residues. Twenty-three, ten and three distinct IgE-binding epitopes were recognized along the amino acid sequence of Ara h 1, Ara h 2 and Ara h 3, respectively. Most of these epitopic stretches contain a high proportion of both electropositively (Arg, Lys) and electronegatively (Asp, Glu) charged residues are thus expected to be nicely exposed at the molecular surface of the allergens. However, a limited number of IgE-binding epitopic regions identified on Ara h 3 is rather intriguing with respect to the high molecular mass and the strong allergenicity associated to this allergen. The Ara h 3 amino acid sequence comprises 507 residues corresponding to a calculated mass close to 58,000 (Rabjohn et al., 1999), and is predicted to occur as a homotrimer similar to that of phaseolin of French bean (Lawrence et al., 1994) and proglycinin A1aB1b of soybean (Adachi et al., 2001), like Ara h 1 (Barre et al., 2005) which also belongs to the vicilin group of seed storage proteins. In addition, native Ara h 3 would occur as a hexameric structure resulting from the stacking of two similarly oriented homotrimers like the glycinin A3B4 homohexamer (Adachi et al., 2003). Apparently, the diversity of the IgE-binding epitopes occurring on the so extended

Abbreviations: Ara h 1, *Arachis hypogaea* allergen 1 (vicilin); Ara h 2, *Arachis hypogaea* allergen 2 (2S albumin); Ara h 3, *Arachis hypogaea* allergen 3 (legumin); TBS, Tris-buffered saline (pH 7.0); TBSTw, Tris-buffered saline (pH 7.0) containing 0.1% (v/v) Tween-20.

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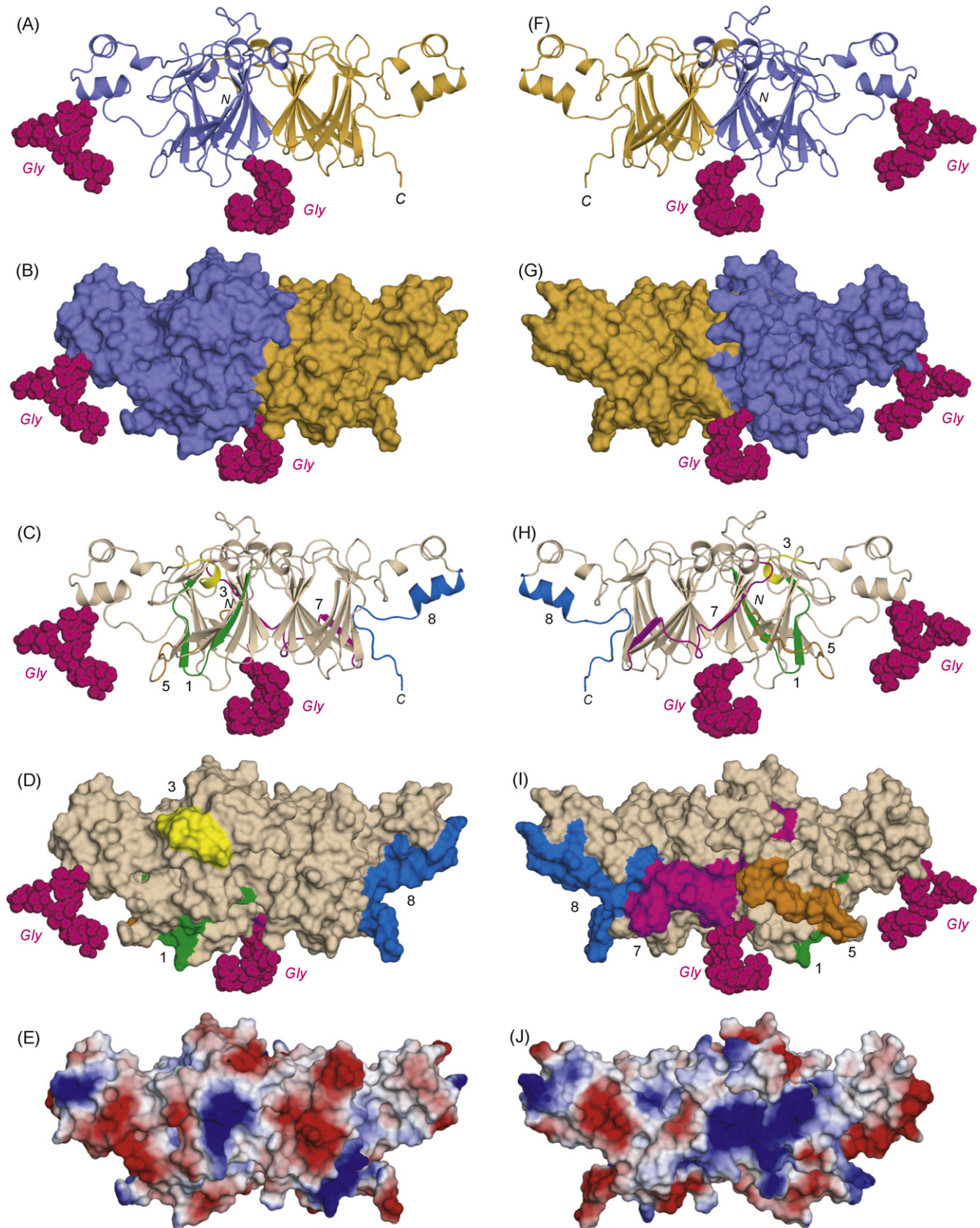


Fig. 1. (A and F) The two faces of the ribbon diagram of the Ara h 3 monomer showing the two cupin motifs colored orange and violet, respectively. The N-glycan chain attached to the monomer is in pink colored CPK. N and C correspond to the N- and C-termini of the polypeptide. (B and G) The two faces of the molecular surface of the Ara h 3 monomer showing the two cupin motifs (colored orange and violet) and the N-glycan chain (colored pink). (C and H) IgE-binding epitopes delineated on the two faces of the ribbon diagram of Ara h 3. Epitopes are numbered and colored green (#1), yellow (#3), orange (#5), magenta (#7) and blue (#8). Regions containing epitopes #2, #4 and

Table 1
Listing of patients allergic to peanut.

Subjects	Code	Sex/age (years)	Allergic history ^a	Symptoms ^b
J.C.	P1	M/6	PA, TNA, EA	
L.A.	P2	M/4	PA, TNA, MUA, EA, MA, FA, P	PR, E
F.E.	P3	F/8	PA, P	E
D.M.	P4	F/6	PA, EA	PR
M.J.	P5	F/6	PA, LA (soybean), EA, P	E
P.A.	P6	M/10	PA, EA, MA, P	A
P.P.	P7	M/11	PA, P	A, PR

^a PA: peanut allergy; TNA: tree nut allergy; LA: legume allergy; MUA: mustard allergy; EA: egg allergy; MA: milk allergy; FA: fish allergy; P: pollinosis.

^b A: asthma; E: eczema; PR: pollinic rhinitis.

molecular surface of Ara h 3 has been underestimated. To clarify this issue, mapping of the IgE-binding epitopic regions on the molecular surface of Ara h 3 was reinvestigated using the Spot technique in combination with a surface analysis of a three-dimensional model built for Ara h 3 by homology modelling.

2. Materials and methods

2.1. Ara h 3 isolation

Ara h 3 was purified from a freshly prepared peanut seed extract according to the procedure described in (Jin et al., 2007). The Ara h 3 preparation was checked for purity by SDS-PAGE. Coomassie blue stained bands were digested with trypsin in the SDS-PAGE gel and mass mapped by MALDI-TOF analysis as previously described (Barre et al., 2007; Barre et al., 2008). The software Protein Prospector was used for the identification of the protein using the NCBI non-redundant database.

2.2. Human sera

Blood samples were drawn after informed consent of seven allergic children and adolescents that have been previously diagnosed as peanut allergic with a convincing clinical history followed by positive skin prick tests and oral food challenges (Table 1). Their reactivity towards Ara h 3 was tested in Western blot experiments using a freshly prepared peanut protein extract in Tris-buffered saline (pH 7.5) as a Ara h 3 source.

2.3. Peptide synthesis and IgE-binding epitope mapping

Overlapping 15-mer peptides, frameshifted by three residues, covering the entire amino acid sequences of Ara h 3 (accession AAM46958) (Viquez et al., unpublished) were prepared by using the Spot technique (Frank, 1992). The protocol previously described in details (Laune et al., 2002) was followed, with the exception of the utilization of the Multi pep automatic Spot synthesizer (Intavis).

The membrane was soaked overnight into 20 mL of Tris-buffered saline (TBS) containing 2 mL blocking buffer (Roche) and 1 g sucrose (pH 7.0), and then washed three times with TBS containing 0.1% (v/v) tween (TBSTw). A 1:10 (v/v) diluted patient serum was added in the presence of an anti-protease cocktail (Roche) and the membrane was incubated in a moist chamber for 2 h. After three washes with TBSTw (pH 7.0) the membrane was stirred in a 1:4000 dilution of mouse monoclonal anti-human IgE labelled with alkaline phosphatase (Sigma) for 1 h. After three

washes with TBSTw (pH 7.0), the interacting peptide spots were colored for 30 min by adding the bromochlorylindolophosphate (BCIP) substrate for alkaline phosphatase (Promega).

Six 15-mer peptides corresponding to the core region of the identified IgE-binding epitopes #1 (peptide P17: 49ALSRLVLRNAL-RRP64), #3 (peptide P65: 193SRRSLPYPSPQT207), #4 (peptide P72: 214REFSPRGQHGRERA228), #5 (peptide P95: 283RILSP-DRKRRQQYER297), #7 (peptide P114: 340ASFKNIGRNRSPDI354), and #8 (peptide P163: 487YGLPREQARQLKNN501), were synthesized by solid-phase synthesis (GenScript Corp., Piscataway, New Jersey, USA) and checked for reactivity towards IgE by ELISA in inhibition experiments.

The capacity of synthetic 15-mer peptides to inhibit the Ara h 3-IgE interaction was checked by ELISA. The wells of a flat-bottomed standard plate (Limbro) were filled with 50 μ L of 10 mM PBS (pH 7.5) containing 5 mg mL⁻¹ of Ara h 3. The plates were incubated overnight at 4 °C and washed three times with PBS (pH 7.5). 100 μ L of PBS (pH 7.5) containing 0.1% Tween 20 (v/v) and 1% BSA (w/v) (PBSTB) were added and the plates were incubated at room temperature for 2 h. 50 μ L of 1:30 diluted patient serum in PBSTB (pH 7.5) previously incubated with 1 or 2 mM of synthetic peptide were added and the plates were incubated for 1 h 30 at room temperature under constant stirring. After three washes with PBSTB, 50 μ L of 1:500 diluted mouse monoclonal anti-human IgE coupled to alkaline phosphatase (Sigma) were added and, after incubation at room temperature for 1 h, three washes were performed with PBSTB. 100 μ L of alkaline phosphatase substrate (Sigma) were added and after incubation for 45 min in the dark, 50 μ L of 3 M NaOH were added to stop the color reaction. The absorbance at 405 nm was recorded on a Titertek Multiscan spectrophotometer. Each value is the mean of three separate experiments. Appropriate blank and substrate controls were performed under the same conditions.

2.4. Molecular modeling

Molecular modeling of the Ara h 3 legumin was carried out on a Silicon Graphics O2 R10000 workstation, using the programs InsightII, Homology and Discover3 (Accelrys, San Diego CA, USA) as previously described in details elsewhere (Barre et al., 2007). Briefly, the X-ray solved cupin structure available at the PDB for the legumin A3B4 glycinin from soybean (PDB code 1OD5) (Adachi et al., 2003), that shares percentages of both identity (from 42% up to 51%) and homology (from 77% up to 86%) with Ara h 3, was used as a template. However, disordered regions for which X-ray coordinates are not available in 1OD5 were omitted in the incomplete model built for Ara h 3. The fold recognition program Phyre (<http://www.sbg.bio.ic.ac.uk/phyre/html/index.html>) yielded a very similar three-dimensional model for the Ara h 3 monomer with, however, a few discrepancies that essentially deal with some extended surface-exposed loops. Ara h 3 was tentatively assigned as a homotrimer displaying an arrangement of the cupin motifs similar to that found in the soybean proglycinin A1aB1b homotrimer (PDB code 1FXZ) (Adachi et al., 2001). Most of the amino acid residues occurring at the interfaces of the cupin motifs of the proglycinin homotrimer are conserved in Ara h 3. Accordingly, no steric clash was observed upon the homotrimeric assembly of the cupin protomers of Ara h 3.

Electrostatic potentials were calculated as previously described (Nicholls et al., 1991) and displayed with GRASP using the parse3 parameters (Gilson and Honing, 1987). The solvent probe radius used for molecular surfaces was 1.4 Å and a standard 2.0 Å-Stern

#6 are lacking on the Ara h 3 model. (D and I) Location of the IgE-binding epitopes on both faces of the Ara h 3 molecular surface (same color code as in C and H). (E and J) Mapping of the electrostatic potentials on both faces of the molecular surface of Ara h 3. Electropositive and electronegative regions are colored blue and red, respectively. Neutral regions are white. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

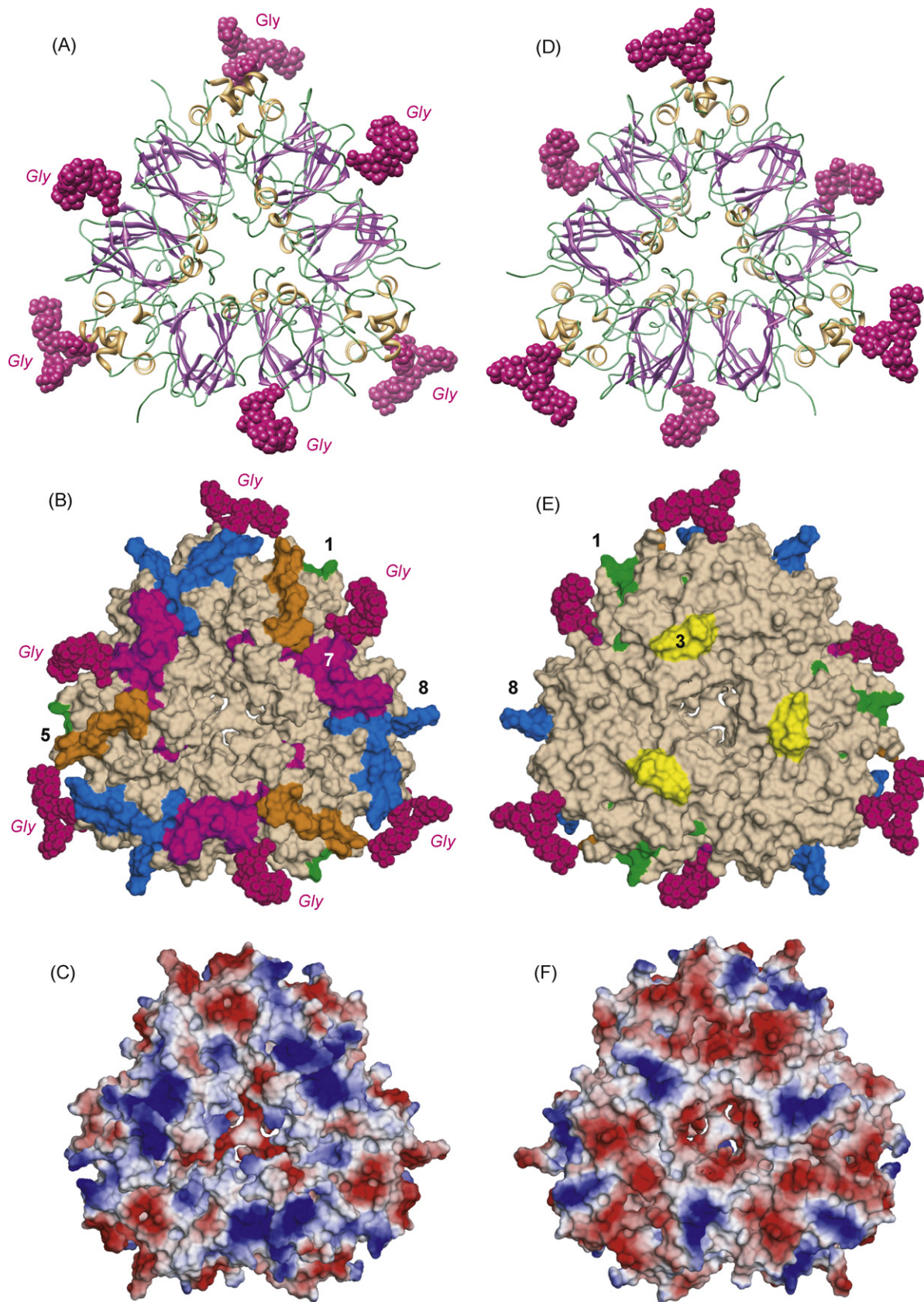


Fig. 2. (A and D) Both faces of the molecular surface (colored wheat) of the Ara h 3 homotrimer. The N-glycan chains attached to the homotrimer is in pink colored CPK. (B and E) Location of the IgE-binding epitopes on both faces of the molecular surface of the Ara h 3 homotrimer (colored wheat). Epitopes are numbered and colored green (#1), yellow (#3), orange (#5), magenta (#7) and blue (#8). Regions containing epitopes #2, #4 and #6 are lacking on the Ara h 3 homotrimer model. (C and F) Mapping of the electrostatic potentials on both faces of the molecular surface of the Ara h 3 homotrimer. Electropositive and electronegative regions are colored blue and red, respectively. Neutral regions are white. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)



Fig. 3. (A) IgE-binding peptides (dark spots) revealed on the Spot membrane. (B) Mapping of the corresponding continuous IgE-binding epitopic regions (black-boxed white letters) along the amino acid sequence of Ara h 3. A similar pattern was obtained with all of the checked IgE-containing sera.

layer was used to exclude ions from the molecular surface. The inner and outer dielectric constants applied to the protein and the solvents were, respectively, fixed at 4.0 and 80.0 and the calculations were performed keeping a salt concentration of 0.145 M.

Surface areas of the Spot characterized linear IgE-binding epitopes on the molecular surface of both the modelled Ara h 3 monomer and homotrimer were calculated using GRASP. The overall conformation of the linear IgE-binding epitopes on the molecular surface was displayed with PyMOL of W.L. DeLano (<http://pymol.sourceforge.net>).

Assuming the putative *N*-glycosylation sites Asn267-Glu-Ser and Asn349-Arg-Ser of Ara h 3 are actually glycosylated by a high-mannose tri-antennary glycan chain with a trimannoside core (Man)₅-(GlcNAc)₂ like in Ara h 1 (van Ree et al., 2000), the glycan chains were built on the Ara h 3 homotrimer using the GlyProt server of Glyco-SCIENCES (<http://www.glycosciences.de/modeling/glyprot/php/main.php>) and represented in CPK on the molecular surface.

The amino acid sequence of Ara h 3 was checked for putative trypsin- and chymotrypsin-cleavage sites by selecting trypsin and chymotrypsin-high specificity (any C-amino acid residue, except for Pro, at N-Trp, -Tyr or -Phe) of the web PeptideCutter program (<http://expasy/tools/peptidecutter>). According to the location of the exposed trypsin and chymotrypsin cleavage sites on the three-dimensional models, both the preserved central core structure and the surface-exposed peeled off regions of the proteins upon the proteolytic attack were identified and mapped on the ribbon diagram of the Ara h 3 monomer.

3. Results

The modelled Ara h 3 monomer exhibits a central core made of two canonical cupin motifs consisting of two bundles of antiparallel β -sheets, associated at both ends to extended loop regions that predominantly contain three short α -helical stretches (Fig. 1A, B, F, and G). Two putative *N*-glycosylation sites Asn267-Glu-Ser and Asn349-Arg-Ser occur along the amino acid sequence of the Ara h 3 monomer. Both *N*-glycosylation sites have been pre-

dicted to be actually glycosylated and a high-mannose *N*-glycan core was built using the GlyProt server and added to the previously built Ara h 3 model (Fig. 1A, B, F, and G). Both faces of the monomer readily differ by their charges, one of them being strongly electronegative (Fig. 1E) whereas the other is essentially electropositive (Fig. 1J). Like soybean glycinin and pea legumin (Adachi et al., 2001), native Ara h 3 consists of a homotrimeric association (Fig. 2A and D) which subsequently occurs as a native hexamer resulting from the non covalent association of two trimers.

Using the Ara h 3-reactive sera from peanut allergic patients as a primary antibody in a Spot experiment, allowed us to identify eight IgE-binding epitopic stretches along the amino acid sequence of Ara h 3 (Fig. 3). They correspond to the continuous amino acid sequence stretches 49ALSRLVLRNALRRP63 (#1), 94EPAQQGRRHQSQRPP108 (#2), 187LRVQQQSRRRSLPYSPPS204 (#3), 217SPRGQHGR225 (#4), 283RLISPD RKRR291, (#5), 319DRRRGRGSR327 (#6), 337ICTASFKNIGNRNSPDIYNP357 (#7) and 490PREQARQLKNNNPFFKFFVPPSEQ513 (#8), respectively. Except for synthetic peptide P17 corresponding to epitope #1 which displayed

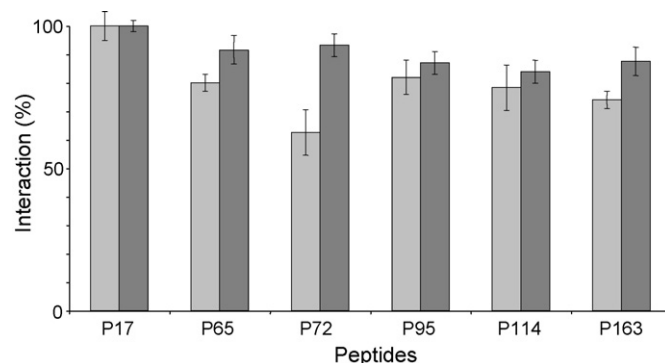


Fig. 4. Inhibition by synthetic peptides P17, P65, P72, P95, P114 and P163 used at 1 mM (grey) and 2 mM (light grey) concentrations of the interaction (expressed as %) of IgE with immobilized Ara h 3. Results are the means of three ELISA measurements.

no inhibition, other peptides corresponding to epitopes #3 (P65), #4 (P72), #5 (P95), #7 (P114) and #8 (P163) 20–25% inhibited the Ara h 3-IgE interaction in ELISA experiment (Fig. 4). Most of these epitopic regions, that roughly amount for 23% of the amino acid sequence, predominantly contain electropositively (**K,R**) charged residues that occur on both faces of the Ara h 3 monomer and display net charges of 2 (#2 and #8) up to 5 (#1) (Fig. 1E and J).

Mapping of the recognized IgE-binding amino acid sequence stretches on the molecular surface of the modelled Ara h 3 monomer revealed a rather good surface exposure for most of them, which exhibit a calculated surface exposed area of 1300 Å² (epitope #7) and 1950 Å² (epitope #8) over a total surface area of 20,255 Å² for the Ara h 3 monomer (Fig. 1C, D, H and I). These surface exposure calculations are not feasible for epitopes #4 and #6 and most

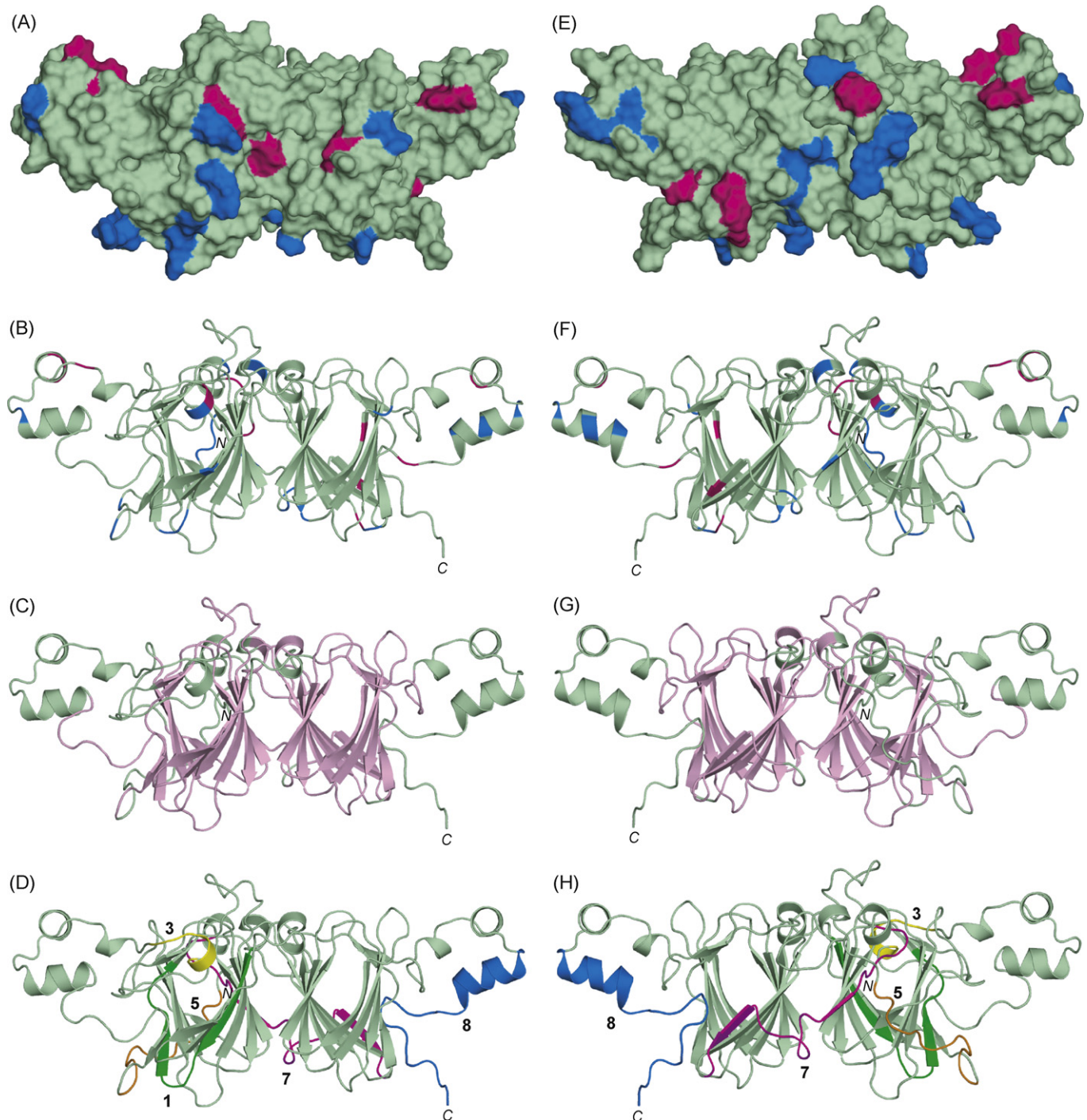


Fig. 5. (A and E) Location of the trypsin (pink) and chymotrypsin (blue) cleavage sites exposed on the molecular surface of both faces of the Ara h 3 monomer. B,F. Location of the trypsin (pink) and chymotrypsin (blue) cleavage sites exposed on both faces of the ribbon diagram of the Ara h 3 monomer. C,G. Ribbon diagram of the preserved core (colored light magenta) of the Ara h 3 monomer. D,H. IgE-binding epitopes delineated on the two faces of the ribbon diagram of Ara h 3. Epitopes are numbered and colored green (#1), yellow (#3), orange (#5), magenta (#7) and blue (#8). Regions containing epitopes #2, #4 and #6 are lacking on the Ara h 3 model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Epitope #1:

Ara h 3	49	A	L	S	R	L	V	L	R	R	N	A	L	R	R	P	63	(5)
Ana o 2	48	A	L	V	R	H	T	I	Q	P	N	G	L	L	L	P	62	(1)
Ber e 2	48	A	A	L	R	N	T	I	R	P	Q	G	L	L	L	P	62	(2)
Cor a 9	53	A	V	I	R	R	T	I	E	P	N	G	L	L	L	P	67	(1)
glycinin B2	47	A	L	S	R	C	T	L	N	R	N	A	L	R	R	P	61	(4)
Fava B4	48	S	L	I	R	R	T	I	D	P	N	G	L	H	L	P	62	(1)
Lupinus 11S	49	A	L	S	R	C	T	I	Q	R	N	G	L	R	R	P	63	(4)
Pisum 11S	47	A	L	S	R	A	T	L	Q	R	N	A	L	R	R	P	61	(4)

Epitope #7:

Ara h 3	337	I	C	T	A	S	F	K	K	N	I	G	R	N	R	S	P	D	I	Y	N	P	357	(3)	
Ana o 2	263	I	C	T	M	R	L	K	E	N	I	I	N	D	P	A	R	A	D	I	Y	T	P	283	(0)
Ber e 2	265	I	C	S	A	T	F	I	Q	N	I	D	N	P	A	E	A	D	F	Y	N	P	285	(-3)	
Cor a 9	304	I	C	S	L	R	L	R	E	N	I	C	T	R	S	R	A	D	I	Y	T	P	324	(1)	
glycinin B2	288	I	C	T	M	R	L	R	Q	N	I	G	Q	N	S	S	P	D	I	Y	N	P	308	(1)	
Fava B4	287	I	C	S	L	K	I	R	E	N	I	A	Q	P	A	R	A	D	L	Y	N	P	307	(1)	
Lupinus 11S	309	L	C	T	M	K	L	R	H	N	I	G	E	S	T	S	P	D	A	Y	N	P	329	(0)	
Pisum 11S	317	V	C	T	A	K	L	R	L	N	I	G	P	S	S	S	P	D	I	Y	N	P	337	(1)	

Fig. 6. Conservation of IgE-binding epitopes #1 and #7 in other tree-nut (Ana o 2, Ber e 2, Cor a 9) and legume (glycinin B1/B2, Fava B4, Lupinus 11S, Pisum 11S) vicilins. Identical residues are in black-boxed white letters, structurally homologous residues (according to the Risler's structural matrix of homology (Risler et al., 1988)) are in grey-boxed black letters. Net charge of the amino acid sequence stretch is indicated into brackets.

of the epitopic stretches #2 and #3 which are lacking on the incomplete model built up for Ara h 3 from the X-ray coordinates of soybean glycinin A3B4. Epitopic stretch #1 consists of a notable exception since it displays a poorly exposed surface area ($460 \text{ \AA}^2$) on the Ara h 3 monomer (Fig. 3D and I) and homotrimer (Fig. 2B and E). In addition, some of these IgE-binding epitopes, e.g. epitopes #7 and #8, are coalescent on the molecular surface of Ara h 3 and should create more extended IgE-binding epitopic areas susceptible to strongly interact with the corresponding IgE antibodies from peanut allergic patients (Fig. 2B and E). This extended epitopic region exhibits a strong electropositive character (Fig. 2C and F).

According to the location of the exposed trypsin and chymotrypsin cleavage sites on the three-dimensional model built for the Ara h 3 monomer (Fig. 5A, B, E, and F), a central core structure remaining unaltered upon the trypsin/chymotrypsin attack is predicted to occur in Ara h 3 (Fig. 5C and G). Interestingly, this core structure contains epitopes #1 and #7 that should remain protected from the proteolytic attack till Ara h 3 is recognized by the immuno-competent cells of the gastro-intestinal tract (Fig. 5D and H). In this respect, epitope #1, which was previously shown to be poorly exposed on the molecular surface of Ara h 3, is almost completely buried into the core structure. Other extra-core epitopes #3, #5 and #8 should be destroyed during the peeling off of Ara h 3 by the digestive proteases.

As shown from multiple amino acid sequence alignments, a few of the IgE-binding epitopes identified on the molecular surface of Ara h 3 display some degree of similarity with the homologous amino acid sequence stretches of other legumin allergens, especially from legume seeds (Fig. 6). Besides these sequence similarities, these regions share a rather similar three-dimensional conformation. As an example, epitope #7 of Ara h 3 exhibits an overall conformation close to that observed in glycinin B1/B2 (A3B4) of soybean (Fig. 7), with electropositive and electronegative residues critical for the interaction with the corresponding IgE paratope located at very similar positions. In addition, the sequence stretch corresponding to epitope #7 of Ara h 3 also contains a putative N-glycosylation site 300Asn-Ser-Ser (Fig. 6). These conformational similarities most probably account for the IgE-binding cross-reactivity previously reported between Ara h 3 and other legumin allergens.

4. Discussion

Using the Spot technique, eight distinct IgE-binding epitopic stretches were identified along the amino acid sequence of Ara h 3. Peptides corresponding to three other amino acid sequence stretches gave a very faint coloration which was considered as being too weak for assessing the epitopic character of these polypeptide regions (see Fig. 3). In addition, these peptides also weakly reacted in negative controls using the 2nd labelled antibody but lacking the 1st IgE antibody. The relevance of these epitopes to Ara h 3 was further checked by their capacity to inhibit the Ara h 3-IgE interaction in ELISA (Fig. 4). Two of the 8 identified epitopes (epitopes #4 and #5) partly overlap with two of the four IgE-binding epitopes previously identified on Ara h 3 using a similar Spot technique (Rabjohn et al., 1999). However, both approaches differ by the fact they used synthetic 15-mer peptides overlapping by eight residues instead of 15-mer peptides overlapping by 12 residues (present work). In addition, their amino acid sequence of Ara h 3 (accession no. AAC63045) slightly differs from the genomic sequence (accession no. AAP46958) (Viquez et al., 2004) we used for delineating the set of peptides to be synthesized for the Spot experiment. This sequence dissimilarity could account for the observed discrepancies.

Some of the eight identified IgE-binding epitopes are well exposed on the molecular surface of the Ara h 3 monomer and dimer and their localization coincide with electropositively charged areas. In this respect, the strongly electronegative face of the Ara h 3 homotrimer only contains the single epitope #3 whereas other epitopes essentially occur on the more electropositively charged face and on the edge of the homotrimer (see Fig. 2B and E). The coalescence of some epitopic regions, e.g. epitopes #7 and #8, should create a more extended epitopic region on the molecular surface of Ara h 3 with an enhanced binding capacity towards a wider repertoire of IgE antibodies.

The allergenic propensity of peanut proteins most probably depends on physico-chemical properties like the resistance to pH and heat denaturation and the resistance to proteases of the gastro-intestinal tract (Mondoulet et al., 2005), by preserving their T and B epitopes till they are recognized by the immuno-competent cells. In this respect, Ara h 3 contains a limited number of trypsin (21 over a total number of 40 putative sites) and chymotrypsin (9 over a total

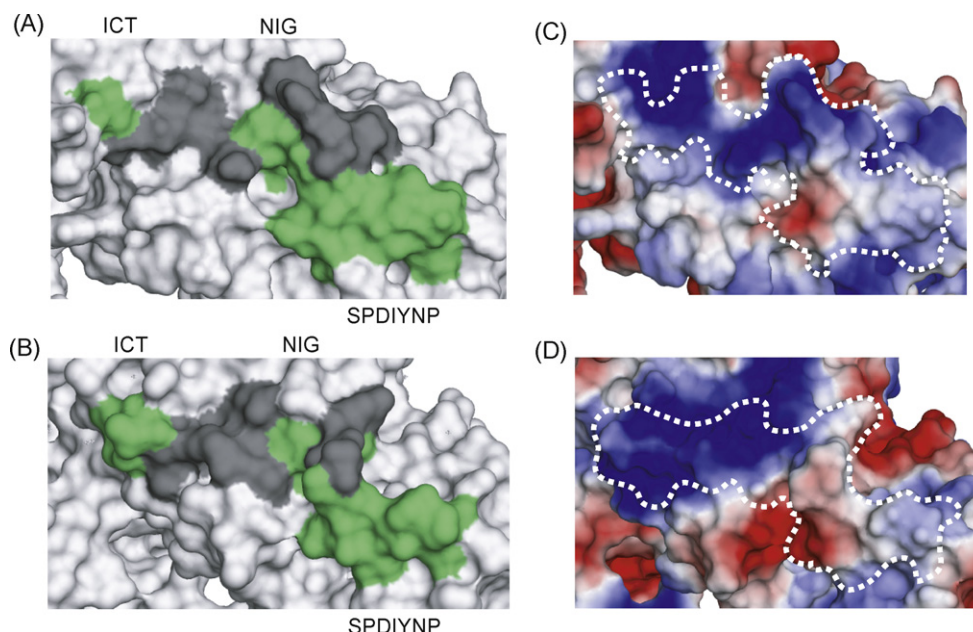


Fig. 7. (A) Overall conformation of epitope #7 on the molecular surface of Ara h 3. The three amino acid residue stretches (ICT, NIG, SPDIYNP) conserved in glycinin B1/B2 of soybean are colored green; other residues are colored grey. (B) Overall conformation of region of glycinin B1 homologous to epitope #7 of Ara h 3. Conserved stretches of residues ICT, NIG and SPDIYNP are colored green, other residues are colored grey. C,D. Mapping of the electrostatic potentials on regions corresponding to epitope #7 of Ara h 3 (C) and homologous region of glycinin B1 (D). Electropositive and electronegative areas are colored blue and red, respectively. Neutral areas are white. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

number of 37 putative sites) cleavage sites sufficiently exposed on the molecular surface to be actually cleaved by the corresponding proteases (Fig. 5A and E). As a result, an extended core structure that amounts for ~60% of the amino acid sequence and essentially corresponds to the tandemly arrayed cupin motifs is predicted to remain unattacked upon digestive proteolysis (Fig. 5C and G). Interestingly, IgE-binding epitopes #1 and #7 occur in the core structure and should remain protected against proteolysis during the gastro-intestinal transit. However, this *in silico* prediction has to be toned down since the susceptibility of Ara h 3 to pepsin, which most probably reduces the core structure by exposing previously buried trypsin/chymotrypsin-susceptible sites, was not taken into account. In agreement with our *in silico* predictions, the proteolysis products of the closely related major allergens Ara h 1 of peanut resulting from an *in vitro* model of gastro-duodenal digestion were demonstrated to retain their allergenicity (Eiwegger et al., 2006; Van Bostel et al., 2008). Obviously, both heat and proteolysis resistance result from a rather packed fold of the secondary structural features in the allergenic proteins and some structural organization patterns, like the cupin motif (Ara h 1, Ara h 3, Ara h 4), seem to be particularly relevant to promote the allergenic potency of protein allergens.

The cupin fold is widely distributed in plant proteins (Dunwell et al., 2004), and many other seed storage legumins have been characterized as potent allergens, especially in tree nuts (Jug r 4 from walnut, Ana o 2 from cashew nut, Ber e 2 from Brazil nut, Cor a 9 from hazelnut) and legume (legumins from pea, lentil and lupine, phaseolin from kidney bean, glycinin B1/B2 from soybean) seeds. IgE-binding epitopic stretches identified along the amino acid sequence of Ara h 3 exhibit no similarity with the corresponding sequence stretches of other legumin allergens except for epitopic stretch #7 which is pretty well conserved in other legumins from legume seeds, especially in glycinin B1/B2 (Fig. 6). Besides this amino acid sequence similarity, this amino acid sequence stretch possesses a closely related overall conformation with charged residues located at equivalent positions

(Fig. 6). Using recombinant soybean glycinin G2-derived polypeptide fragments (Beardslee et al., 2000) identified a IgE-binding dominant epitope 219SGFAPEFLKEAFGVN233 glycinin G2 shares with Ara h 3. This amino acid sequence stretch partly coincides (underlined) with one (237GNIFSGFTPEFLAQ251) of the four IgE-binding epitopes previously identified on Ara h 3 by the Bannon's group (Rabjohn et al., 1999). However, in our hands, the synthetic peptides used in Spot experiments gave no reaction with the IgE-containing sera from allergic patients. Eleven IgE-binding epitopes have been previously identified along the amino acid sequence of glycinin G2 from soybean (Helm et al., 2000). Some of them, e.g. epitopes #5, #6 and #11 partly coincide with epitopes #3, #5 and #8 of Ara h 3, respectively. Similarly, the IgE-binding epitope 231IVKVKDELVRVPSRS247 identified in the legumin allergen Ana o 2 (Wang et al., 2003) partly overlaps epitope #5 of Ara h 3 (283RILSPDRK291). These epitopic communities could account for the IgE-binding cross-reactions that have been reported to occur between peanut and other tree nuts and legume seeds (Asero et al., 2004; Goetz et al., 2005; Magni et al., 2005).

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