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RESEARCH ARTICLE

Trypsin resistance of the major peanut allergen Ara h 6 and allergenicity of the digestion products are abolished after selective disruption of disulfide bonds

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Scope: 2S-albumins Ara h 2 and Ara h 6 are the most widely recognized and potent allergens for peanut-allergic patients. These allergens are particularly resistant to proteolysis and the digestion products generally retain significant allergenicity. Five disulfide bridges (DB) stabilize Ara h 6 overall structure and their influence on the trypsin resistance and on the allergenicity of the digestion products was investigated.

Methods and results: Progressive disruption of each DB was performed by site-directed mutagenesis. Successful refolding of Ara h 6 variants was confirmed by circular dichroism. Trypsin resistance, IgE-binding capacity and allergenic potency, as assessed by *in vitro* mediator release assay with sera from peanut-allergic patients, was not affected by the deletion of the C-terminal DB at Cys^{84}-Cys^{124}. Additional disruption of DB at Cys^{14}-Cys^{71} or at Cys^{73}-Cys^{115} rendered $Arg^{16/20}$ or Arg^{114} susceptible to trypsinolysis, respectively, but affected principally the IgE-binding capacity of Ara h 6. DB disruption at Cys^{26}-Cys^{58} or at Cys^{59}-Cys^{107} led to an extensive proteolytic degradation and a complete loss of allergenic potency of the digestion products.

Conclusion: Selective disruption of the DB stabilizing the protease-resistant core of Ara h 6 eliminated the IgE-binding capacity of the trypsin-degradation products and their ability to trigger mast cell degranulation.

Keywords:
2S-albumin / Allergenicity / Disulfide bridges / Peanut / Trypsin resistance

1 Introduction

Peanut allergy represents a major threat of severe IgE-mediated reactions for children and adults [1–3]. It generally persists through adulthood, thus affecting approximately 1% of the total population [4, 5]. Recent studies on oral immunotherapy using crude peanut extract (CPE) provided promising results. However, use of hypoallergenic variants of peanut allergens has been further proposed to perform specific immunotherapy without the risk to expose patients to serious side-effects after CPE administrations [6–11].

Among the 11 allergens so far identified in peanut, Ara h 2 and Ara h 6 have been recently described as the most frequently recognized allergens by IgE antibodies from peanut-allergic patients and as the most potent effectors, accounting for up to 90% of the allergenic potency in CPE [12–17]. Ara h 2 and Ara h 6 belong to the family of 2S-albumins, particularly present in legumes, tree nuts and seeds. These proteins of low molecular weight possess a core structure stabilized by a network of four conserved disulfide bridges (DB) [18]. During seed maturation, most of 2S-albumins are processed in two large and small subunits with a molecular weight of 8–10 and 3–5 kDa, respectively [18, 19]. However, processing of 2S-albumins in peanut appears to be rather ineffective. Indeed,

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Abbreviations: CD, circular dichroism; **DB**, disulfide bridge; **MS**, mass spectrometry; **r**, recombinant; **red**, reduced and *S*-alkylated; **RBL**, rat basophilic leukemia

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while a small amount of processed Ara h 6 coexists with the full-length protein, Ara h 2 does not seem to be degraded by peanut proteases [16, 20]. Nevertheless, the 'typical' pattern of processed 2S-albumin in two small and large subunits can be observed with native Ara h 2 or Ara h 6 after trypsin digestion [21, 22]. The domain located between the two subunits is thereby particularly susceptible to proteolytic attack, even when Ara h 2 and Ara h 6 are properly folded. Otherwise, the compact three-dimensional structure is preserved by the DB network that provides a great resistance not only towards trypsin but also towards pepsin thus allowing Ara h 2 and Ara h 6 to reach almost intact the gut immune system [14, 22, 23]. Indeed, even after cleavage between the large and small subunits, the two fragments remain associated by two DB, thus forming a heterodimer as immunoreactive as the intact protein [16, 21].

The conformational structure of Ara h 2 and Ara h 6 plays a critical role in the allergenicity. The development of functional tests using fluid-phase IgE binding, thus preserving allergen conformation, revealed that DB disruption by complete reduction and S-alkylation of Ara h 2 and Ara h 6 alters drastically the IgE-binding capacity and the allergenic potency [16, 24–27]. The relative importance of conformational epitopes compared to sequential ones was also demonstrated [26]. However, while characterization of linear B-cell epitopes is exhaustively undertaken, the role of the conformational epitopes in 2S-albumin allergenicity still needs to be investigated [18, 28–30]. Moreover, the design of novel hypoallergenic variants, which is generally based on the disruption of B-cell sequential epitopes, could be improved by considering the conformational epitopes accounting for the allergenic properties of peanut allergens [31].

We previously reported the cloning and the expression of a recombinant (r) Ara h 6 protein that, after correct refolding, exhibited an immunoreactivity similar to that of the natural counterpart [32]. In the present work, the impact of Ara h 6 conformation and stability on trypsin resistance and allergenicity of the breakdown products was addressed by studying the relative contribution of each DB.

2 Materials and methods

2.1 Allergic patients

Ten children were recruited at the Paediatric Allergy Clinic of Hôpital Necker-Enfants Malades (Paris, France, Table 1). According to their medical history, symptoms involved skin, respiratory tract, gastrointestinal tract and cardiovascular system. Peanut allergy was confirmed by positive open oral food challenge and by high concentrations of serum Ara h 6-specific IgE. Agreements were received from the parents to perform scientific investigations.

2.2 Reagents and materials

Unless otherwise stated, all reagents were of analytical grade from Sigma (St. Louis, MO). Reduction and S-alkylation of Ara h 6 was performed as previously described in order to prepare denatured Ara h 6 ($H6_{red}$) [16]. Labelled Ara h 6 was prepared by covalent linkage of the native protein to the tetrameric form of acetylcholinesterase [33].

2.3 Site-directed mutagenesis

Recombinant Ara h 6 variants, with Cys-Ala substitutions leading to the disruption of the corresponding DB, were generated by using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The plasmid pET9c-PSC33 that contains the *ara h 6* gene [32], was used as template for PCR amplifications with Pfu polymerase in the presence of sense and antisense primers as listed in Table 2. After site-directed mutagenesis, the entire coding region of *ara h 6* was verified by DNA sequencing in order to confirm Cys-Ala substitutions.

2.4 Protein expression, refolding and purification

Overnight cultures of recombinant *Escherichia coli* BL21(DE3) were used to inoculate fresh medium at a dilution of 1:40.

Table 1. Serological and clinical data of the peanut-allergic patients

Patient #	Age (year)	Sex	Clinical signs (medical history)	Open OFC threshold (mg)	Ara h 6-specific IgE (IU/mL)
51	5	M	V, GU	400	700
470	4	M	A, R, U	6000	800
263	10	F	A, V, CP	800	440
134	1.5	M	QO, GU, V	ND	150
110	8	M	A, U, V	200	46
39	6	M	A, R, U	50	160
102	11	M	QO, GU, V	ND	170
430	4	M	R	400	25
443	12.5	M	A, RC, V	1600	100
496	5.5	M	A, GU	1600	87

M, male; F, female; A, asthma; CP, cutaneous pruritus; GU, generalized urticaria; QO, Quincke's oedema; R, rhinitis; RC, rhino conjunctivitis; U, urticaria; V, vomiting; OFC, oral food challenge (threshold dose eliciting reactions); ND, not done (risk of anaphylactic shock).

Table 2. Strategy used for the disruption of disulfide bridges in Ara h 6

Constructs	Removed disulfide bridges	Mutagenic primers (Sequences [5'→3'])
H6-5	***C84A*** + ***C124A***	**C84A** → CATGGAAAACCAG**GCC**GACCGTCTGCAGGACCG
		C124A → CGTTTCTGGTGGTCGT**GCC**TAATAGGATCCGGCTGC
H6-5.1	*C84A* + *C124A* ***C14A*** + ***C71A***	**C14A** → GGTGACTCTTCTTCT**GCC**GAACGTCAGGTTGACC
		C71A → GGAAAACACTGAACGC**GCC**ATGTGCGAAGCTCTGC
H6-5.2	*C84A* + *C124A* ***C26A*** + ***C58A***	**C26A** → GTTAACCTGAAACCG**GCC**GAACAGCACATCATGC
		C58A → GACCAGCAGCAGCGT**GCC**TGCGACGAACTGGACC
H6-5.3	*C84A* + *C124A* ***C59A*** + ***C107A***	**C59**A → CAGCAGCAGCGTTGC**GCC**GACGAACTGGACCAGATGG
		C107A → GAACCTGCCGCAGCAG**GCC**AACTTCCGTGCTCCG
H6-5.4	*C84A* + *C124A* ***C73A*** + ***C115A***	**C73A** → CACTGAACGCTGCATG**GCC**GAAGCTCTGCAGCAG
		C115A → CGTGCTCCGCAGCGT**GCC**GACCTGGACGTTTCTGG
H6-5.1.4	*C84A* + *C124A* *C14A* + ***C71A*** ***C73A*** + *C115A*	**C71A+C73A** → GAAAACACTGAACGC**GCC**ATG**GCC**GAAGCTCTGCAGCAG
H6-5.2.3	*C84A* + *C124A* *C26A* + ***C58A*** ***C59A*** + *C107A*	**C58A+C59A** → GACCAGCAGCAGCGT**GCCGCC**GACGAACTGGACCAGATGG

Substitutions of Cys by Ala residue are indicated by the codon GCC bolded and underlined.

Cultures were grown at 37°C until the optical density at 600 nm reached 0.5. The expression of the recombinant proteins was then induced by adding isopropylthio-β-D-galactoside (1 mM) for 5 h. After centrifugation, bacterial pellets were stored at −20°C until extraction.

Frozen pellet corresponding to 100 mL of culture was resuspended in 10 mL of Tris buffer (0.1 M, pH 8) containing different protease inhibitors (bacitracin 50 μg/mL, benzamidine 300 μg/mL, leupeptin 20 μg/mL, chymostatin 20 μg/mL, pepstatinA 2.5 μg/mL, AEBSF 60 μg/mL). After sonication (60 s) and centrifugation (10 min, 10 000 × *g*, 4°C), the pellet was resuspended in 10 mL of extraction buffer (Tris 0.1 M, pH 8, guanidine-HCl 6 M, DTT 5 mM) in order to solubilize the inclusion bodies for 2 h on a rotary mixer at room temperature. Suspensions were then centrifuged (10 min, 10 000 × *g*, 4°C). As recombinant proteins are produced without tag, the supernatant containing the whole protein content of solubilized inclusions bodies was immediately submitted to the refolding protocol as previously described [32]. The full-length refolded rAra h 6 was then purified using RP-HPLC. Purified fractions were analyzed by gel electrophoresis under reducing conditions and by mass spectrometry before being freeze-dried in order to confirm that rAra h 6 was not degraded by bacterial proteases. Recombinant protein was resuspended in potassium phosphate buffer (0.1 M, pH 7.4) and total protein concentration was determined with the BCA protein assay (Pierce Biotechnology, Rockford, IL 61105).

2.5 Trypsin digestion

Influence of DB disruptions on Ara h 6 stability was addressed by testing its resistance towards proteolysis. Although Ara h 6 is also resistant to pepsin action, we chose to perform trypsin digestion of the different mutants. Indeed the potential trypsin-cleavage sites are distributed almost evenly in Ara h 6 sequence, especially in the N-terminus part of the protein, and they are more numerous than the potential pepsin-cleavage sites, as estimated with ExPASy PeptideCutter tool [34]. Recombinant Ara h 6 variants were thus subjected to trypsin digestion (Promega, 18000 U per mg of enzyme) in Tris buffer (20 mM, pH 8). One mg of Ara h 6 was incubated with 50 μg of trypsin at 37°C under agitation for 4 h. The reaction was stopped by adding protease inhibitors and samples were stored at −20°C until further characterization. Digestion pattern was visualized by gel electrophoresis using 3 μg of protein resuspended in Laemmli buffer with β-mercaptoethanol.

2.6 Circular dichroism spectroscopy

Circular dichroism (CD) analysis of the recombinant proteins (0.1 mg/mL in potassium phosphate buffer (20 mM, pH 7.4)) was performed at 20°C on a JASCO-810 spectropolarimeter using 0.1 cm path length cells. The spectrum was recorded from 190 to 250 nm at a scanning speed of 100 nm/min with a 1 s time constant, a 0.1 nm resolution and a 2 nm constant

band pass. Three spectra were accumulated for each construct. The averaged spectrum was corrected by subtracting the baseline spectrum obtained under identical conditions with buffer alone. The results are expressed as mean residue ellipticity (deg.cm^2.dmol^{-1}).

2.7 Mass spectrometric characterization

Mass determination was carried out using a matrix-assisted laser desorption ionization-time-of-flight instrument (MALDI-TOF, Voyager DE RP apparatus, PE Biosystems, France). Mass spectrometry analysis was performed on samples resuspended in carbonate buffer (50 mM, pH 8.5) under reducing and non-reducing conditions. Reducing treatment was performed with β-mercaptoethanol (250 μM) at 95°C for 10 min. The different samples were then diluted in 0.2% TFA solution just before mass spectrometry analysis. Samples were mixed in a 1:1 ratio with a matrix solution of α-cyano-4-hydroxycinnamic acid or sinapinic acid. Analysis was performed in reflector or linear negative mode.

2.8 IgE-immunoreactivity analysis

The IgE-binding capacity of the different variants was assessed by evaluating their capacity to inhibit IgE binding of labelled Ara h 6 in a reverse enzyme allergo-sorbent test (EAST inhibition), as previously described [35]. Results were expressed as B/B_0 where B_0 and B represented the amount of Ara h 6 linked to immobilised IgE antibodies in the absence or presence of a known concentration of recombinant Ara h 6 variants, respectively. The IgE reactivity of competitors was calculated with the concentration inhibiting 50% of the IgE binding to Ara h 6 (IC50).

2.9 Mediator release assay

RBL SX -38 cells, rat mast cells expressing α, β and γ chains of the human high-affinity receptor FcεRI for IgE, were used to perform degranulation tests, as previously described [24]. Cells were passively sensitized with human IgE antibodies immunopurified from a pool of 40 different sera from peanut-allergic patients (see Supporting Information Table S1). Mediator release, induced by incubation with different concentrations of recombinant Ara h 6 variants, was determined by measuring the enzymatic activity of β-hexosaminidase. Results were expressed as percentage of the reference release after activation of the sensitised cells with anti-human IgE (LE27 clone; 100 ng/mL).

2.10 Statistical analysis

Data were analyzed using the non-parametric Wilcoxon matched pairs signed rank test. Statistical analyses were performed with GraphPad Prism 5.01 software and $p < 0.05$ was considered significant ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

3 Results

3.1 Cloning and expression of Ara h 6 variants

As Ara h 6 possesses an additional DB (DB5) compared to the four canonical cystines in 2S-albumins (Fig. 1), the mutational approach was initiated by removing DB5 at Cys^{84}-Cys^{124}, thus leading to the variant H6–5 (Table 2). The other four DB were then additionally removed in the corresponding variants H6–5.1, H6–5.2, H6–5.3 and H6–

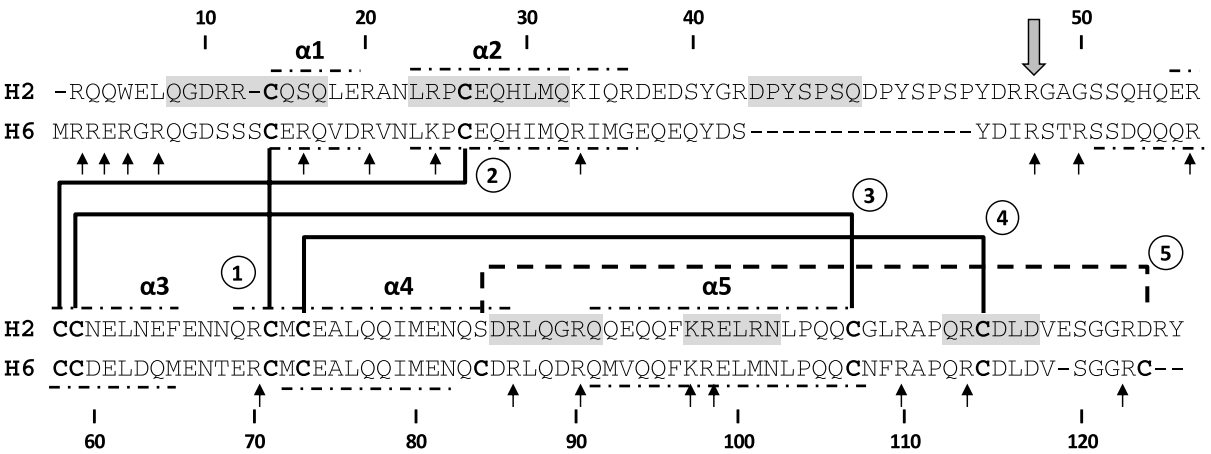

Figure 1. Alignment of Ara h 2 and Ara h 6 and schematic representation of the disulfide bond pattern conserved in 2S-albumins. Dotted line indicates the additional disulfide bridge, DB5, in Ara h 6 compared to Ara h 2. Arrows point to tryptic cleavage sites. Grey arrow indicates the cleavage site processed in vivo by peanut protease for Ara h 6 [16, 20] or in vitro by trypsin for Ara h 2 or Ara h 6, thus leading to the 'typical' pattern of processed 2S-albumins with two small and large subunits [21]. Linear IgE-binding epitopes in Ara h 2 are grey-shaded [26, 30, 39]. The five α-helices are represented by dashed lines. Numbering of Ara h 6 residues is indicated.

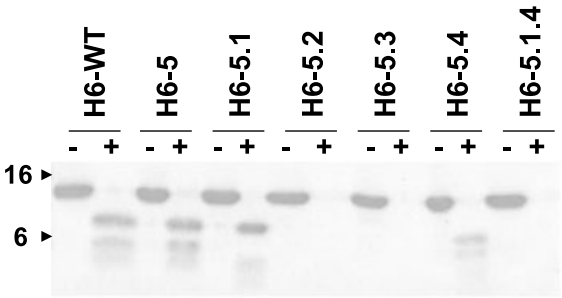

Figure 2. Resistance of Ara h 6 variants to digestion by trypsin. Intact and digested protein samples were separated by SDS-PAGE under reducing conditions.

5.4. Finally, three different DB were removed in the variants H6–5.1.4 and H6–5.2.3. For these constructs, in addition to DB5, the disrupted DB were selected in regard to the structural proximity of Cys^{58} and Cys^{59} engaged in DB2 and DB3, and Cys^{71} and Cys^{73} engaged in DB1 and DB4, respectively (Fig. 1).

Regarding the expression level of the recombinant proteins and their purification, no significant differences were observed between the different variants (data not shown). As observed on SDS-PAGE analysis (Fig. 2), the Ara h 6 variants were purified to near homogeneity. The average yield of recombinant allergen after refolding and purification was around 1 mg/L of culture.

3.2 Refolding of Ara h 6 variants

Spectroscopic analysis of secondary structure of the different Ara h 6 variants was performed using CD spectroscopy. The far UV spectra of most variants displayed a profile similar to that observed with rAra h 6 (Fig. 3). In this case, spectra revealed a maximum at around 192 nm, a minimum at 208 nm and a shoulder near 222 nm. These spectra, characteristic of 2S-albumins, underlined the predominance of helical structures in Ara h 6. Only variant H6–5.2.3 and the reduced and S-alkylated Ara h 6 ($H6_{red}$) exhibited a CD spectrum characteristic of a random coiled nature.

3.3 Role of the disulfide bridges on the resistance of Ara h 6 to trypsin hydrolysis

Trypsinolysis of rAra h 6 led to the disappearance of the initial band at 14 kDa. The 'typical' pattern of naturally processed 2S-albumins was then observed with two large and small subunits of 10 kDa and 5 kDa, respectively. Identification of the tryptic products by MALDI-TOF MS localized the cleavage sites at Arg^{5}-Gly^{6}, Arg^{7}-Gln^{8}, Arg^{47}-Ser^{48} and Arg^{50}-Ser^{51} (Fig. 1 and Table 3). Under non-reducing conditions, the small (f6/8–47) and large (f51–124) subunits of rAra h 6 remained associated by two disulfide bonds, DB1 and DB2

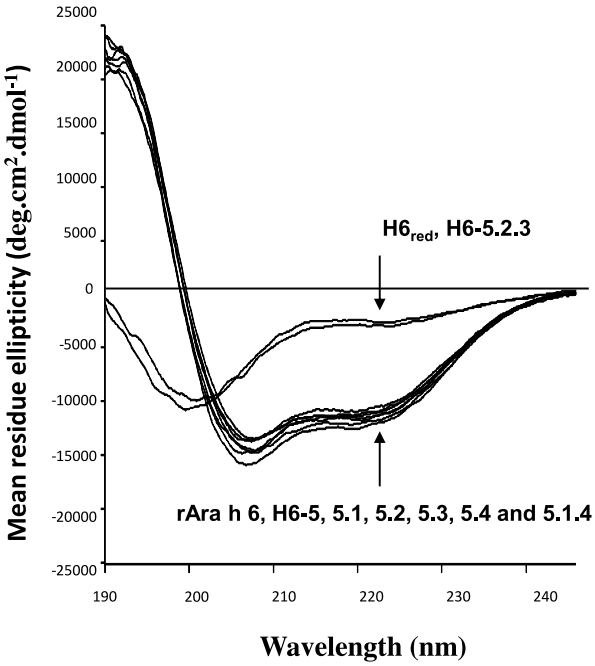

Figure 3. Far UV circular dichroism spectra of the different Ara h 6 variants and the denatured allergen ($H6_{red}$).

(Fig. 1), as confirmed by MS with an apparent mass of 13 787 Da (Table 3).

The removal of DB5 did not affect the degradation pattern of Ara h 6 as confirmed by MS analysis. In contrast, the additional deletion of DB2 or DB3 reduced drastically trypsin resistance of H6–5.2 and H6–5.3, as evidenced by the absence of heterodimeric structure (Fig. 2). In fact, most of the potential cleavage sites were processed by trypsin and among the numerous fragments detected by MS, the largest one (f58–86/90) exhibited a mass of 3.9 kDa. The deletion of DB1 or DB4 was less detrimental for Ara h 6 resistance since heterodimeric structures were still detected after trypsinolysis. Disruption of DB1 exposed peptide bonds Arg^{16}–Gln^{17} and Arg^{20}-Val^{21} to trypsin attack and resulted in an heterodimer containing a truncated small subunit (f17/21-47). Furthermore, the heterodimer persisting after trypsinolysis of H6–5.4 exhibited a truncated large subunit (f51-114) resulting from the cleavage after Arg^{114} (Fig. 1). Disruption of three DB induced the complete degradation of H6–5.1.4 (Fig. 2) and H6–5.2.3 (data not shown). Accordingly, reduction of the five DB led to a similar extensive degradation of $H6_{red}$ by trypsin (data not shown).

3.4 Role of the disulfide bridges on IgE-binding capacity of Ara h 6

rAra h 6 and the variants H6–5, H6–5.1, H6–5.2 and H6–5.3 displayed similar capacity to inhibit the binding of IgE antibodies from patient #443 to Ara h 6 (Fig. 4A). In contrast, IgE reactivity was substantially decreased for H6–5.4

Table 3. Identification by MS of the heterodimeric structures observed after trypsin digestion

Constructs (mass, Da)	Masses observed after trypsinolysis		
	Under non-reducing conditions	Under reducing conditions (fassigned peptide sequence)	
		Small subunit	Large subunit
rAra h 6 (14 842)	13 787	4986 (f6-47)	8800 (f51-124)
	13 554	4774 (f8-47)	
H6-5 (14 772)	13 479	4986 (f6-47)	8736 (f51-124)
		4774 (f8-47)	
H6-5.1 (14 709)	12 519	3824 (f17-47)	8700 (f51-124)
	12 021	3325 (f21-47)	
H6-5.2 (14 710)	7306[a)]		3418 (f58-86)
	6852[a)]		3932 (f58-90)
H6-5.3 (14 712)	7412[a)]		3418 (f58-86)
	6965[a)]		3929 (f58-90)
H6-5.4 (14 712)	12 493	4758 (f8-47)	7733 (f51-114)

a) Products corresponding to heteropolymeric structures.

and drastically reduced for H6–5.1.4, H6–5.2.3 and $H6_{red}$, as revealed by much higher IC50. Influence of DB4 disruption on IgE reactivity was confirmed by testing sera from nine other patients (Fig. 5A). While IgE-binding capacity of Ara h 6 remained not affected by DB5 disruption, it was significantly decreased after the additional deletion of DB4, but also of DB1. Accordingly, IgE reactivity of H6–5.1.4 was further reduced but not totally abolished after the simultaneous disruption of DB1 and DB4. While deletion of either DB2 or DB3 did not affect significantly the IgE-binding capacity, simultaneous disruption of DB2 and DB3 led to a drastic loss of IgE reactivity.

Considering serum from patient #443, tryptic fragments from rAra h 6 and H6.5 displayed an inhibition capacity similar to that found before trypsinolysis (Fig. 4B). After analysis of ten patients' sera, digestion of rAra h 6 and H6–5 appeared to induce a slight reduction of their IgE-binding capacity, with IC50 only 2 to 3-fold higher than those determined for undigested variants (Fig. 5A and 5B).

Compared to rAra h 6 tryptic fragments, the digestion products of H6–5.1 displayed a ten-fold reduced IgE reactivity (Fig. 5B). Digestion of H6–5.4 resulted in a more substantial decrease but the digestion products retained a significant IgE reactivity with half of the tested sera (Fig. 5B).

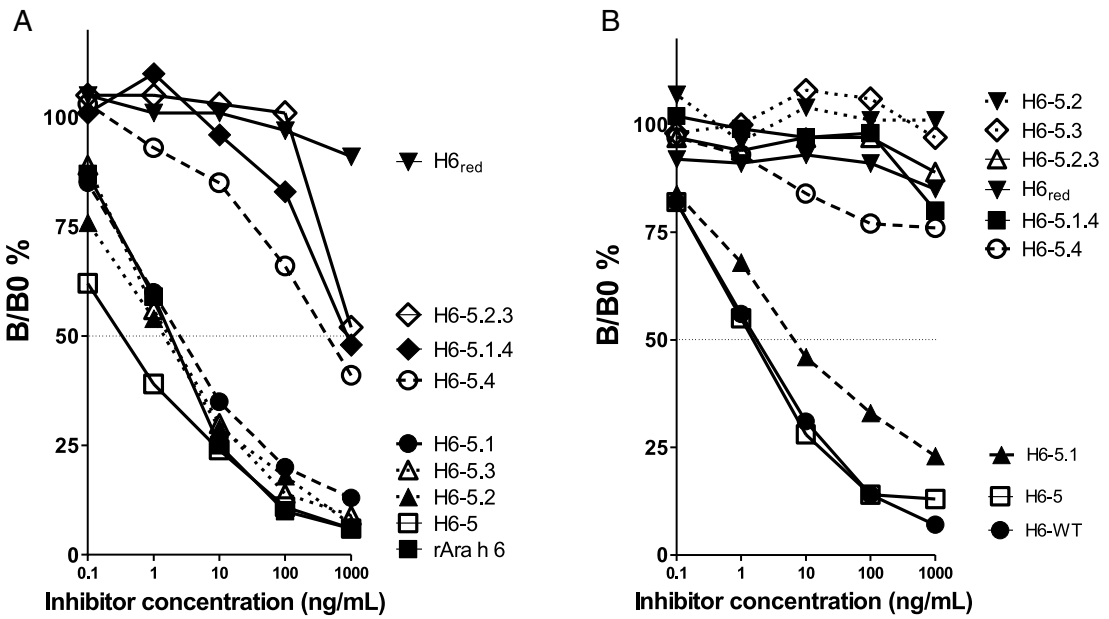

Figure 4. Competitive binding-inhibition (EAST inhibition) of IgE antibodies from patient #443 to Ara h 6 by recombinant Ara h 6 variants and the denatured allergen ($H6_{red}$), before (A) and after (B) trypsin digestion.

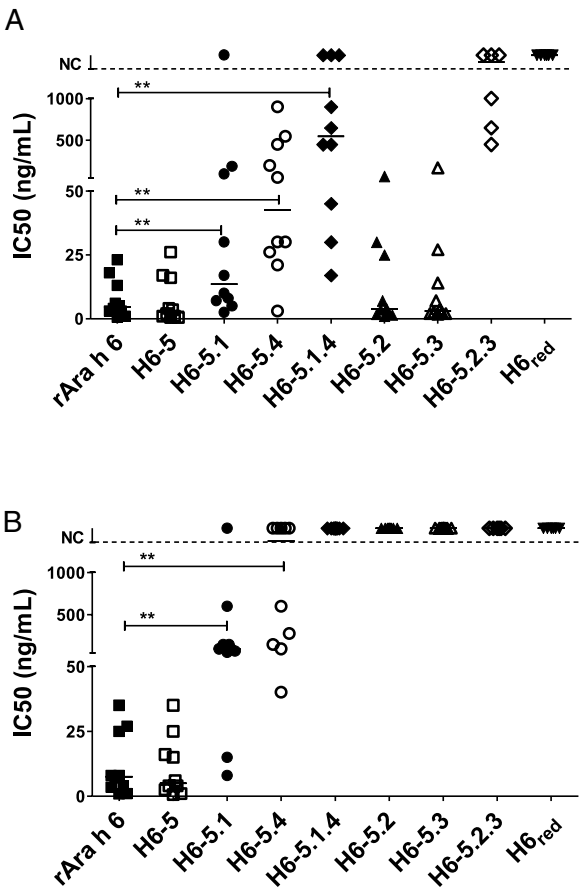

Figure 5. Competitive binding-inhibition (EAST inhibition) of IgE antibodies from ten peanut-allergic patients to Ara h 6 by recombinant Ara h 6 variants and the denatured allergen ($H6_{red}$), before (A) and after (B) trypsin digestion. 50% inhibitory concentration (IC50) were determined. NC, not calculable.

No IgE-binding capacity was detected with tryptic fragments from other variants.

3.5 Role of the disulfide bridges on Ara h 6 capacity to trigger RBL SX-38 degranulation

The allergenic potencies of Ara h 6 variants missing one or two DB were comparable (Fig. 6A). A substantial decrease in the maximal release was observed only with the variant H6–5.1.4 while H6–5.2.3 or $H6_{red}$ were not able to induce significant RBL SX-38 degranulation.

Even after trypsinolysis, rAra h 6 and H6–5 remained potent elicitors of RBL SX-38 degranulation (Fig. 6B). In fact, tryptic fragments of rAra h 6 or H6–5 were as efficient as the undigested proteins. The tryptic fragments of H6–5.1 and H6–5.4 were also able to trigger *in vitro* degranulation although the concentration of digestion products required for inducing the maximal release was approximately ten-fold higher than that measured for rAra h 6. On the other hand, trypsin digestion of H6–5.2, H6–5.3 and H6–5.1.4 totally abrogated their capacity to induce RBL SX-38 degranulation.

4 Discussion

In certain allergens displaying conformational IgE epitopes, the disruption of intramolecular DB has been shown to induce protein unfolding and decrease of the allergenicity [31, 36–38]. Reduction and *S*-alkylation of 2S-albumins further demonstrated the importance of the DB network on the susceptibility to proteolytic digestion and on the persistence of large digestion products even after prolonged hydrolysis [22]. Moreover, reduced and S-alkylated Ara h 2 is unable to trigger effector cell degranulation [26]. Accordingly, in this work, reduced Ara h 6 could not induce RBL SX-38 degranulation or exhibit any IgE-binding capacity. The role of each DB on Ara h 6 susceptibility to trypsinolysis and on the allergenic potency of tryptic fragments was therefore investigated.

The non-canonical DB5 at Cys^{84}-Cys^{124} was first removed. Although DB5 links the C-terminus of Ara h 6 to the compact fold, its disruption did not affect trypsin resistance or IgE reactivity, thus confirming that DB5 does not contribute to protein stability as no regular secondary structure is associated with the Asn^{108}-Cys^{124} domain.

Deletion of DB1 (Cys^{14}-Cys^{71}) rendered only two additional peptidic bonds, Arg^{16}-Gln^{17} and Arg^{20}-Val^{21}, susceptible to trypsin attack. Interestingly, the domain between Gln^{8} and Gln^{17} corresponds to an immunodominant IgE-binding epitope in Ara h 2 [26, 30, 39]. Linear IgE-binding epitopes in Ara h 6 are not yet described. However, considering the similar context of exposure of these domains on the surface of Ara h 2 and Ara h 6 (Fig. 1 and [26]), the domain Gln^{8}-Gln^{17} could be an IgE-binding epitope in Ara h 6 as well. The reduced IgE-reactivity of H6–5.1 may thus result from the substitution of Cys^{14} by Ala in this putative epitope. However, as sequential epitopes require also an appropriate structural context for proper interactions with IgE antibodies [26], the modified exposure of domain Gln^{8}-Gln^{17}, which leads to the cleavage at Arg^{16} and Arg^{20}, may also impair its recognition by IgE antibodies. Accordingly, trypsin cleavage of the N-terminal part of the small subunit at $Arg^{16/20}$ led to a further decrease of IgE reactivity, thus correlating with the presence of an immunodominant IgE-binding epitope. In the same way, disruption of DB4 (Cys^{73}-Cys^{115}) permitted the cleavage after Arg^{114} and led to a substantial decrease of IgE reactivity. Here also, Cys^{115} may be involved in a putative IgE-binding domain between Gln^{113} and Glu^{118}, as reported in Ara h 2 [30]. In addition, DB4 connects α4 and α5 helices that flank the 'hypervariable region' between Gln^{83} and Gln^{91}, which is an immunodominant IgE-binding epitope of numerous 2S albumins [18]. Disruption of DB4 could thus disturb the correct exposure of epitopes located in the hypervariable region and in the C-terminal domain. Finally,

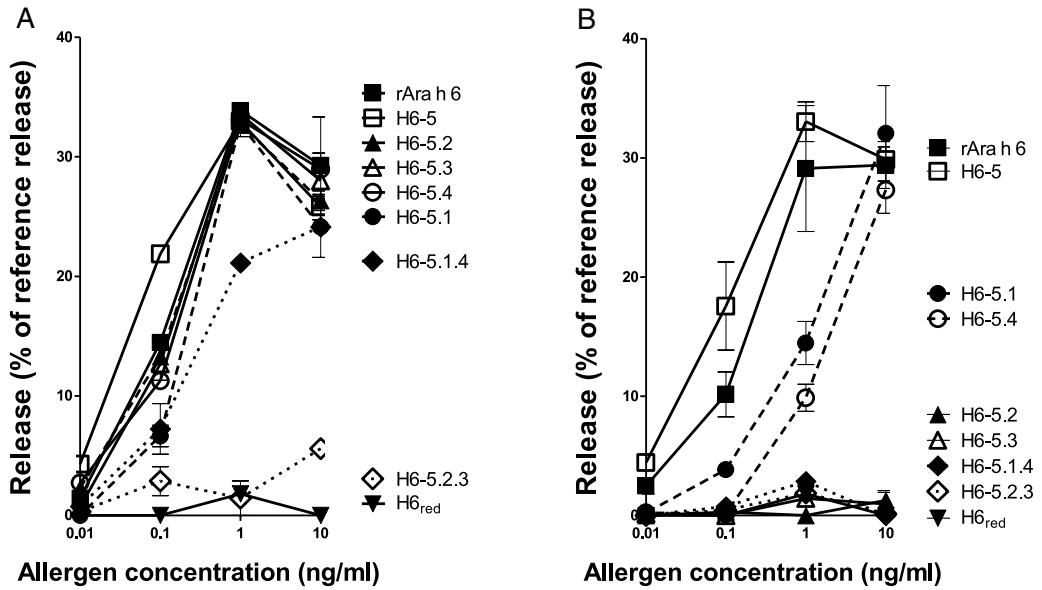

Figure 6. Mediator release assay with RBL SX-38 cells passively sensitized with immunopurified human IgE antibodies from a pool of 40 peanut-allergic patients (Supporting Information Table S1) in response to increasing concentrations of recombinant Ara h 6 variants and the denatured allergen ($H6_{red}$), before (A) and after (B) trypsin digestion.

when DB1 and DB4 were simultaneously removed, the IgE reactivity was further decreased. The heterodimer was no more observed after trypsinolysis, thus revealing that DB1 and DB4 somewhat contribute to the protease-resistance core of Ara h 6. However, despite the disruption of three DB out of five, CD analysis of H6–5.1.4 did not reveal drastic destabilization of the secondary structures, thus suggesting that the remaining DB2 and DB3 play predominant roles in the maintenance of the global fold of Ara h 6.

This was further confirmed with H6–5.2 and H6–5.3 variants. Indeed, disruption of DB2 or DB3 dramatically decreased trypsin resistance of Ara h 6. In particular, the peptidic bond Arg^{57}-Cys^{58} on α-helix α3 became susceptible to trypsin attack. Simultaneous deletion of both DB2 and DB3 also precluded the correct refolding of Ara h 6. DB2 and DB3 thus seem to play pivotal roles in the structural integrity of the hydrophobic core, which has been described to involve the α-helices α2, α4 and α5 [21]. As DB2 and DB3 connect α3 to α2 and α5, respectively (Fig. 1), α3 seems to be involved as well in the stabilization of Ara h 6 core. Surprisingly, IgE reactivity was not affected by the removal of DB2 or DB3, thus suggesting that the structural constraints imposed by DB2 and DB3 do not interfere with the exposure of immunodominant IgE epitopes and that Cys^{26}, Cys^{58}, Cys^{59} and Cys^{107} are not critical residues for IgE-binding.

Despite the reduced IgE reactivity of H6–5.1 and H6–5.4, the disruption of one or two DB did not affect the allergenic potency of Ara h 6. Even after the disruption of three DB, the variant H6–5.1.4 still displayed substantial allergenic potency. Such discrepancies between IgE-binding capacity and allergenic potency were also observed when comparing digested and undigested Ara h 6, or Ara h 2 [21]. In fact, the capacity to trigger RBL cell degranulation correlated with the presence of the heterodimeric structure in the digestion products, as observed with H6–5.1 and H6–5.4 digesta. Accordingly, digestion products of H6–5.2 and H6–5.3, with fragments smaller than 4 kDa, did not exhibit any allergenic potency thus confirming that the efficient cross-linking between receptor-bound IgE antibodies required a relatively preserved Ara h 6 core.

In conclusion, our results provide a better understanding of the role of each DB on the conformation of Ara h 6, on its resistance to trypsin and on its allergenicity. It would be now of great interest to extend this approach to other 2S-albumins, and, particularly to Ara h 2. The cross-reactivity between the two allergens could then be further investigated in order to identify common epitopes and determine the relative importance of sequential epitopes compared to conformational ones. In parallel with the mutagenesis of residues critical for the recognition of sequential epitopes by IgE antibodies, the targeted disruption of DB could be of great interest for the design of hypoallergenic variants, not only for specific immunotherapy applications but also for the generation of hypoallergenic seeds containing 2S-albumins partially destabilized with only one or two DB disruptions and consequently more digestible.

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