Allergenicity of peanut component Ara h 2: Contribution of conformational versus linear hydroxyproline-containing epitopes

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Key messages:

Absence of hydroxyproline in recombinant Ara h 2 may affect the accuracy of component-resolved diagnostics.

Short peptides of Ara h 2 encompassing linear hydroxyproline-containing epitopes can trigger RBL mast cell degranulation.

Relative contributions of linear and conformational epitopes to Ara h 2 allergenicity are variable among peanut-allergic patients.

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Capsule summary: Small fragments comprising the hydroxyproline-containing domain of Ara h 2 can trigger RBL mast cell degranulation and may serve to improve the accuracy of peanut allergy diagnosis.

Key words: Food allergy, peanut allergen, post-translational modifications, hydroxyproline, IgE-binding, conformational and linear epitopes.

Abbreviations used: rec: recombinant, r/a: reduced and S-alkylated, pep: peptide, P^{OH}: hydroxyproline, CD: circular dichroism, RBL: Rat Basophilic Leukemia.

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ABSTRACT

Background: 2S-albumin Ara h 2 is the most potent peanut allergen and a good predictor of clinical reactivity in allergic children. Post-translational hydroxylation of proline residues occurs in DPYSP^{OH}S motifs, which are repeated two or three times in different isoforms.

Objectives: We investigated the impact of proline hydroxylation on IgE-binding and the relative contributions of linear and conformational epitopes to Ara h 2 allergenicity.

Methods: Peptides containing DPYSP^{OH}S motifs were synthesized. A recombinant variant of Ara h 2 without DPYSP^{OH}S motifs was generated by deletion mutagenesis. IgE reactivity of 18 French and 5 American peanut-allergic patients toward synthetic peptides and recombinant allergens was assessed by IgE-binding inhibition assays and by degranulation tests of humanized rat basophilic leukemia cells.

Results: Hydroxyproline-containing peptides exhibited an IgE-binding activity equivalent to that of the unfolded Ara h 2. In contrast, corresponding peptides without hydroxyproline displayed a very weak IgE-binding capacity. Despite removal of the DPYSP^{OH}S motifs, the deletion variant still displayed Ara h 2 conformational epitopes. The IgE-binding capacity of Ara h 2 was then recapitulated with an equimolar mixture of a hydroxylated peptide and the deletion variant. Hydroxylated peptides of 15 and 27 amino acid residues were also able to trigger cell degranulation.

Conclusions: Sensitization toward linear and conformational epitopes of Ara h 2 is variable among peanut-allergic patients. Optimal IgE-binding to linear epitopes of Ara h 2 requires post-translational hydroxylation of proline residues. The absence of hydroxyproline could then affect the accuracy of component-resolved diagnostics using recombinant Ara h 2.

INTRODUCTION

Peanut is one of the most common causes of severe allergic reactions to food [1;2]. The IgE-mediated peanut allergy affects more than 1% of the children and is outgrown by only 20% of the patients [3].

Until now, twelve allergens, *e.g*. Ara h 1 to 13, with Ara h 3/4 describing the same protein, have been identified in peanut (*Arachis hypogea*)[4]. Ara h 1, 2 and 3 were initially recognized as the major peanut allergens [5-7]. Recently, Ara h 2 and Ara h 6 were described as being the most clinically relevant peanut allergens as observed *in vitro* with effector cell-based assays and *in vivo* with skin prick test [8-17]. Accordingly, the IgE response to Ara h 2 has been reported to be a good predictor of clinical allergy in children [18;19] and the IgE response to Ara h 6 could also provide good diagnostic performance [20;21].

Ara h 2 and Ara h 6 belong to the 2S-albumin family. They share a compact conformation characterized by five α-helical structures and stabilized by a network of four conserved disulfide bridges [22]. This arrangement provides a core structure highly resistant to proteolysis so that treatment of 2S-albumins with digestive enzymes does not affect significantly their allergenicity [23-27]. Ara h 2 and Ara h 6 are 59% homologous but compared to Ara h 6, two insertions of 14 and 26 amino acid residues occur in Ara h 2 major isoforms, *i.e.* Ara h 2.01 and Ara h 2.02 [6;28]. These insertions are exposed on a flexible surface loop and comprise the repeated DPYSP^{OH}S motif, with the second proline being hydroxylated [23;29]. The DPYSP^{OH}S-containing domain has been reported to be a major linear IgE-binding epitope [30;31]. The impact of proline hydroxylation on Ara h 2 IgE reactivity has not yet been investigated, although this post-translational modification has been shown to influence the IgE-binding to Phl p 1, a major allergen from timothy grass pollen [32]. On the other hand, conformation of 2S-albumins is also essential for the allergenic potency since suppression of disulfide bridge formation in Ara h 2 and Ara h 6 by chemical reduction or by site-directed mutagenesis reduced their IgE reactivity significantly [26;30;33-35]. In this regard, some studies reported a predominance of IgE recognition of conformational epitopes on Ara h 2 while others suggested a higher proportion of IgE-binding to linear epitopes [30;33;36].

In the present work, we aimed to determine the relative contributions of linear and conformational epitopes to the allergenic potency of Ara h 2. For this purpose, we first characterized the IgE-reactivity of a stably unfolded Ara h 2 and of a properly refolded recombinant Ara h 2. Discrepancies of IgE-binding capacity between the recombinant and native allergens led us to investigate particularly the influence of proline hydroxylation. We therefore compared the IgE reactivity of 18 French and 5 American peanut-allergic patients toward native and recombinant Ara h 2 and toward synthetic peptides containing DPYSP^{OH}S motifs with or without hydroxyproline. Moreover, considering that the DPYSP^{OH}S-containing domain is located on a flexible surface loop in Ara h 2 and is absent in Ara h 6, this domain was not expected to contribute significantly to the stability of the global fold of 2S-albumins [23;37]. We thus generated a recombinant variant of Ara h 2 lacking the DPYSP^{OH}S motifs in order to investigate the contribution of conformational epitopes to the IgE-reactivity of Ara h 2 without any IgE-binding to the linear hydroxyproline-containing epitopes.

METHODS

Human sera

French sera for this retrospective study were collected from 18 peanut-allergic children recruited at the Paediatric Allergy Clinic of Hopital Necker-Enfants Malades after informed consent from patient's parents (see Table E1 in the Online Repository and [38]). All serum samples were collected during routine clinical practice and were studied in accordance with the purpose of the initial study. Based on their medical history, symptoms of the IgE-mediated peanut allergy involved skin, respiratory tract, gastrointestinal tract and cardiovascular system. Five sera from American peanut-allergic patients with a strong history of peanut-induced immediate hypersensitivity and peanut-specific IgE ≥ 13 KAU/L (ImmunoCap, Phadia; Uppsala, Sweden) in serum were collected within 6 months of this study (see Table E2 in the Online Repository). All adult patients and the parents or guardians of minors signed informed consent. Minors who were >6 years of age, signed an assent. The University of Colorado Denver Institutional Review Board approved this study.

Allergen preparations

The 2S-albumins Ara h 2 and Ara h 6 were purified from whole peanut protein extract prepared with commercially roasted peanuts (Virginian variety) as previously described [35]. Separation of the different isoforms of Ara h 2 is described in the Online Repository.

The gene encoding Ara h 2.01 (Swiss-Prot accession number Q6PSU2-2, Fig. 1) was synthesized by using codons optimized for bacterial expression (Genscript USA Inc., Piscataway, NJ, USA) and inserted into the *E.coli* expression plasmid pET9c (Novagen-Merck, Damstadt, Germany). The variant recAra h 2.Δ was obtained by replacing the domain GRDPYSPSQDPYSPSP of recAra h 2.01 by the dipeptide DS naturally occurring in Ara h 6 (Fig. 1). Expression, purification and refolding of recombinant proteins are described in the

Online Repository. Refolding of the recombinant proteins was verified by circular dichroism (CD) spectroscopy as previously described [26].

Reduction and S-alkylation of Ara h 2 (all isoforms) and Ara h 6 was performed as previously described in order to prepare stably unfolded 2S-albumins [35].

Peptides

Several peptides comprising major linear IgE-binding epitopes, as initially reported by Stanley *et al* [31], were synthesized by taking into account the post-translational hydroxylation of proline residues. The peptide (pep) 1-21: *RQQWELQGDRRCQSQLERANL* covered the N-terminal part of Ara h 2 (Fig. 1). The peptide containing two hydroxyprolines (pep 2P^{OH}) corresponded to the domain found in Ara h 2.01 isoform: *DPYSP*^{OH}*SQDPYSP*^{OH}*SPY*. The peptide containing three hydroxyprolines (pep 3P^{OH}) corresponded to the domain found in Ara h 2.02 isoform: *DPYSP*^{OH}*SQDPYSP*^{OH}*SQDPDRRDPYSP*^{OH}*SPY* (Fig. 1). Corresponding peptides control without hydroxyproline (pep 2P and pep 3P) were also synthesized. The different peptides were purified by RP-HPLC and analysed by MALDI-TOF (see in the Online Repository). Characterization of the synthetic peptides by CD spectroscopy did not reveal a significant presence of α-helix or β-sheet secondary structures (Fig. E1). Gel permeation chromatography did not evidence the formation of peptide aggregates under physiological conditions (Fig. E2).

IgE-immunoreactivity analysis

In agreement with the assumption from Albrecht *et al.* that “fluid-phase binding of IgE antibodies is more relevant in relation to *in vivo* allergenicity” [30], we developed a reverse enzyme allergo-sorbent test that is not based on the binding capacity of allergens immobilized on solid phase but measures the binding of labelled allergens by patients’ IgE antibodies captured by an anti-human IgE monoclonal antibody immobilized on the solid phase [39]. In this test, plates were first coated with anti-human IgE monoclonal antibody LE27. Fifty µL/well of serum from each patient at adequate dilutions were incubated overnight at 4°C. After washing, 25 µL of inhibitors (*i.e.* increasing concentrations of the tested molecule) and 25 µL of labelled native Ara h 2 were mixed and incubated for 4 h at room temperature. Labelled Ara h 2 (all isoforms) used for this IgE-binding assay were prepared by covalent linkage of the native protein to the tetrameric form of acetylcholinesterase (AChE) [40]. Ellman's reagent was then used as AChE chromogenic substrate and absorbance at 414 nm was measured [41]. Results were expressed as B/B0. B0 and B represent the amount of labelled Ara h 2 bound to immobilised IgE antibodies in the absence or presence of a known concentration of inhibitor, respectively. The concentration inhibiting 50% of the IgE binding to labelled allergen (IC50) was evaluated by using GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA, USA).

Mediator release assay

Degranulation assay was performed with rat basophilic leukemia (RBL) SX -38 cells as previously described [9]. Cells were passively sensitized with IgE antibodies immunopurified from indivividual serum as previously described [9]. Mediator release was induced by incubation with different concentrations of synthetic peptides, native or recombinant allergens and was determined by measuring the β-hexosaminidase activity. Results were expressed as a percentage of the reference release induced with anti-human IgE (LE27 clone; 100 ng/mL).

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.

RESULTS

Impact of reduction and alkylation on Ara h 2 IgE-reactivity

We first wanted to evaluate the loss of IgE-reactivity induced by chemical reduction of the disulfide bridges and the resulting suppression of the conformational epitopes. Stable unfolding of Ara h 2 was performed by reduction and alkylation (r/a). The unfolded state of r/a Ara h 2 was confirmed by CD spectroscopy with a single spectrum minimum close to 200 nm instead of the two broad minima at 208 and 222 nm, typical for α-helical secondary structures largely present in native Ara h 2 (Fig. 2). This denaturing treatment reduced considerably the IgE-binding capacity of Ara h 2 (Fig. 3). However, for 9 of 18 patients (sera 313, 576, 101, 102, 486, 572, 841, 109 and 907), r/a Ara h 2 retained a significant IgE-reactivity with an IC50 ranging from 0.5 to 750 nM. Two patients (109 and 907) even displayed an IgE response to r/a Ara h 2 comparable to that against the native allergen. Of note, the isoform Ara h 2.02 displayed a slightly but significantly higher IgE-binding capacity than Ara h 2.01 ($p=0.0003$). As illustrated in Fig. 4A, with four representative sera, and in Fig. E3, the difference of IgE-reactivity between Ara h 2.01 and Ara h 2.02 increased concomitantly with the IgE-binding capacity of r/a Ara h 2.

IgE-binding capacity of native and recombinant Ara h 2

We then wanted to assess whether a recombinant form of Ara h 2.01 shared similar allergenic properties with its native counterpart. It thus appeared that recombinant and native Ara h 2 displayed different patterns of IgE-reactivity among the patients (Fig. 4A and Fig. E3). Interestingly, the IgE-reactivity of recAra h 2.01 was inversely proportional to that of r/a Ara h 2. While native and recombinant Ara h 2 were similarly bound by IgE antibodies from patient 847, r/a Ara h 2 was not recognized, thus indicating that most of Ara h 2-specific IgE antibodies from patient 847 recognized conformational epitopes. In this regard, recAra h 2.01 also appeared to be properly refolded, as confirmed by CD analysis with the predominance of α-helical structures (Fig. 2). Conversely, recAra h 2.01 was poorly recognized by IgE antibodies from patient 907 whereas r/a Ara h 2 displayed an IgE reactivity almost as high as that of the native allergen. In this case, most of Ara h 2-specific IgE antibodies from patient 907 recognized linear epitopes. Patients 432 and 841 displayed intermediate IgE-reactivity toward recAra h 2.01 and r/a Ara h 2 (see also Fig. E3). It was then noteworthy that even when recombinant and native Ara h 2 were reduced and alkylated, the IgE-binding capacity of the recombinant allergen remained lower than that of the native form. The influence of post-translational modifications that naturally occurs in peanut seeds but not in prokaryotes was then further investigated.

Impact of proline hydroxylation on Ara h 2 IgE-reactivity

Four peptides containing two or three DPYSP^{OH}S motifs, as found in Ara h 2.01 and Ara h 2.02 respectively, were synthesized with or without hydroxyprolines (Fig. 1). As illustrated by patients 841 and 907, peptides with hydroxyprolines displayed an IgE-binding capacity at least 1000-fold higher than that of peptides without hydroxyprolines (Fig. 4B). Inhibition of IgE-binding to Ara h 2 was always more efficient with the 27-AA-long peptide 3P^{OH}, with three DPYSP^{OH}S motifs, than with the 15-AA-long peptide 2P^{OH}, with only two DPYSP^{OH}S motifs. Surprisingly, the IgE-binding capacity of the 27-AA-long peptide was as high as that of the full-length r/a Ara h 2 for all the tested sera (Fig. 4B and Fig. E3), thus suggesting that pep 3P^{OH} was bound by nearly all of the IgE antibodies recognizing linear epitopes. The peptide 1-21 did not exhibit any significant IgE-binding capacity for any of the French sera.

Evaluation of the relative contribution of linear *vs* conformational epitopes to Ara h 2 IgE-binding capacity

In order to determine the contribution of conformational epitopes to the IgE-reactivity, a recombinant variant of Ara h 2 lacking the DPYSP^{OH}S motifs, recAra h 2.Δ, was generated by deletion mutagenesis (Fig. 1). Suppression of this disordered domain did not prevent recAra h 2.Δ to refold properly (Fig. 2). Furthermore, recAra h 2.Δ and recAra h 2.01 were bound by IgE antibodies with an apparent similar affinity, thus confirming that most of the conformational epitopes were preserved on recAra h 2.Δ (Fig. 4C).

IgE-binding to conformational epitopes could then be specifically inhibited with the deletion variant recAra h 2.Δ and IgE-binding to linear epitopes could be inhibited with the synthetic peptide pep 3P^{OH}. Accordingly, while IgE-binding to Ara h 2 was only partially inhibited by pep 3P^{OH} or recAra h 2.Δ separately, an equimolar mixture of pep 3P^{OH} and recAra h 2.Δ exhibited an inhibitory capacity similar to that of the native Ara h 2 (Fig. 4C). The relative contribution of linear and conformational epitopes to the IgE-reactivity of Ara h 2 was then evaluated for each patient. For patient 847, around 82% of the IgE-binding to Ara h 2 was due to the recognition of conformational epitopes while around 87% of the IgE-binding to Ara h 2 was due to the recognition of linear epitopes for patient 907 (Fig. 4C). Five sera from American peanut-allergic patients were similarly tested (Fig. E4). As observed with French patients, hydroxylation of the synthetic peptides was required to obtain significant IgE-binding to the synthetic peptides. IgE-binding to linear epitopes also appeared to be restricted to the DPYSP^{OH}S-containing domain. Of note, pep 1-21 displayed a rather significant IgE-binding capacity for one serum but still with a much lower affinity than pep 3P^{OH} (serum D119, Fig. E4A). American patients also displayed variable levels of sensitization toward linear and conformational epitopes of Ara h 2 (Fig. E4B).

Allergenic activity of the DPYSP^{OH}S-containing peptides

The capacity of the different variants and synthetic peptides from Ara h 2 to cross-link IgE/FcεRI complexes was evaluated with a degranulation assay of RBL SX-38 cells. As expected, when cells were passively sensitized with immunopurified IgE antibodies from patient 847, the synthetic peptides did not display any allergenic activity and only properly folded allergens, *i.e.* native and recombinant Ara h 2, were able to induce cell degranulation (Fig. 5). In this case, we also confirmed that the synthetic peptides did not possess any intrinsic ability to induce mediator release and that basophil degranulation was actually dependent on the presence of specific IgE antibodies. Indeed, when cells were loaded with IgE antibodies from patient 907, the peptides 3P^{OH} and, to a lesser extent, 2P^{OH} could trigger cell degranulation almost as efficiently as the full-length allergen. Unfolded r/a Ara h 2 also retained a strong allergenic potency. In contrast, the synthetic peptides without hydroxyproline pep 3P and pep 2P (not shown) did not display any allergenic potency. The pattern of cell degranulation induced with IgE antibodies from patient 432 and 841 were in line with IgE-binding assays, since potency of the hydroxylated peptides correlated with that of r/a Ara h 2 and with a decreasing potency of the recombinant allergens (Fig. 5).

DISCUSSION

Being the most potent allergens from peanut, 2S-albumins Ara h 2 and Ara h 6 are attractive target molecules for therapeutic applications. Treatment of peanut-allergic mice with purified Ara h 2 and 6 has been recently shown to provide an equivalent level of desensitization than with a crude peanut extract [(14)]. The use of stably unfolded 2S-albumins has also been proposed for the development of safer immunotherapeutic treatments [(26;42)]. However, the presence of major linear IgE-binding epitopes persisting in unfolded allergens could limit the advantages of such allergoids. In this regard, determination of the relative importance of linear and conformational IgE-binding epitopes in Ara h 2 allergenicity could be of interest in order to optimize specific immunotherapy.

As previously observed with Ara h 6, reduction and alkylation of Ara h 2 led to a substantial decrease of the IgE-binding capacity [(26;33)]. Nevertheless, approximately 50% of the tested sera exhibited a significant IgE-reactivity toward r/a Ara h 2. Similarly, Starkl *et al.* observed that the residual IgE-binding capacity of r/a Ara h 2, compared to untreated Ara h 2, was highly variable among peanut-allergic patients [(34)]. In contrast, we never evidenced such residual IgE-reactivity for r/a Ara h 6 when using the same competitive fluid-phase assay [(26)]. Even the patients displaying the highest IgE-reactivity to r/a Ara h 2 did not recognize r/a Ara h 6, thus suggesting that only Ara h 2 contains immunodominant linear IgE-binding epitopes (Fig. E5).

For the first time, post-translational modifications were shown to be critical for IgE-binding to Ara h 2 linear epitopes and explained the differences of IgE-reactivity between native and recombinant Ara h 2 [(23)]. We thus demonstrated the importance of proline hydroxylation in the motif DPYSP^{OH}S, which was, in our experimental setup, the sole major linear IgE-binding epitope of Ara h 2. Accordingly, the inhibitory capacity of r/a Ara h 2 was completely recapitulated with a single peptide containing three DPYSP^{OH}S motifs. Although domain 1-21 was reported to contain immunodominant IgE-binding epitopes [30], the corresponding synthetic peptide rarely display a significant inhibitory capacity. The weak IgE-binding capacity of r/a recAra h 2 also confirmed the absence of major linear epitope when prolines are not post-translationally modified. This result is in agreement with Albrecht *et al.* who reported that a mix of IgE-binding Ara h 2-derived peptide did not interfere detectably with the IgE-binding to a recombinant form of Ara h 2 [30]. In contrast, Bublin *et al.* observed that a considerable proportion of IgE binding to Ara h 2, Ara h 1 and Ara h 3 could be inhibited with a mix of three synthetic peptides containing the N-terminal region of Ara h 2 and the repeated motif DPYSPS without hydroxylated proline [36]. A weak IgE-reactivity of the non-hydroxylated peptide pep 3P was also detected with patients 841 and 907. However, the clinical relevance of this low affinity binding is questionable since pep 3P displayed no allergenic potency when pep $3P^{OH}$ induced cell degranulation.

Another important finding was thereby the ability of the 15- and 27-residues long peptides, pep $2P^{OH}$ and $3P^{OH}$, to induce basophil degranulation. This result was rather unexpected for such short peptides, especially when considering that these peptides did not form aggregates under physiological conditions (Fig. E2), as previously reported for other allergenic peptides less than 3 kDa [43;44]. The allergenic activity of these peptides thus suggests that their repeated $DPYSP^{OH}S$ motifs can be bound simultaneously by at least two IgE antibodies. Considering the minimum peptide size that could optimally cross-link IgE/FcεRI complexes, as calculated by Bannon *et al.* with data from Kane *et al.* [45;46], the tri-valent peptide pep $3P^{OH}$ was just long enough to induce an efficient cell degranulation. Surprisingly, with only 15 amino acid residues, the bi-valent peptide pep $2P^{OH}$ still displayed an allergenic activity, albeit much lower than that of pep $3P^{OH}$. Thus, accordingly to its higher valency, pep $3P^{OH}$ displayed a higher IgE-binding capacity and a higher allergenicity than pep

$2P^{OH}$, which is in agreement with previous studies suggesting a higher allergenicity of native Ara h 2.02 compared to Ara h 2.01 [31;47].

The allergenic potency of the $DPYSP^{OH}S$-containing peptides provides also new insight into the residual allergenicity of hydrolyzed peanut proteins. It has been previously shown that Ara h 2 digested with trypsin/chymotrypsin displayed minimal reduction in IgE binding capacity and allergenicity [23]. Recently, Shi *et al.* reported that even with an extensive reduction in the size of the IgE-binding peptides and a substantial decrease of IgE-binding capacity, peanut flour hydrolysates still displayed high allergenic potency, certainly because of Ara h 2 fragments [27]. Accordingly, our data showed that any peptide containing more than one $DPYSP^{OH}S$ motif, even smaller than 3 kDa, could induce mast cell degranulation and thereby present an allergenic risk. The preparation of hydrolysate as an alternative to native peanut flour proteins in immunotherapy could then take into account the specific detection of such short peptides. Moreover, as illustrated with patient 432, even when the contribution of linear epitopes to Ara h 2 allergenicity is not predominant, r/a Ara h 2 and pep $3P^{OH}$ could still present an anaphylactic risk (Fig. 5). In this regard, the use of hypoallergens without hydroxyproline such as r/a recAra h 2 or r/a recAra h 2.Δ may be preferred to unfolded native allergens for the development of future specific immunotherapy.

Finally, the fact that the immunodominant linear IgE-binding epitopes of Ara h 2 are almost exclusively located in the $DPYSP^{OH}S$-containing domain permitted us to use the peptide pep $3P^{OH}$ and the recombinant variant Ara h 2.Δ to quantify the relative contribution of linear and conformational epitopes to the IgE-binding. The level of sensitization to linear and conformational epitopes appeared then to be quite variable among patients and we did not evidence a predominant IgE-recognition of a certain type of epitopes over the other one. In fact, when testing two different pools of sera from French or American peanut-allergic patients, the contributions of linear and conformational epitopes to the IgE-binding capacity of Ara h 2 were globally equivalent in both cases (data not shown). In this regard, comparison of Ara h 2 IgE-reactivity between sera from French and American peanut-allergic patients did not reveal any significant differences. The absence of proline hydroxylation in recombinant Ara h 2 could then significantly affect the accuracy of component-resolved diagnostics for most peanut-allergic patients by under-estimating the IgE response to Ara h 2. Recently, Lin *et al.* developed a bioinformatics approach to identify patients with symptomatic peanut allergy using peptide microarray immunoassay. The use of hydroxylated peptides as biomarkers could also certainly increase the prediction performance. Moreover, sensitization to linear epitopes has been associated with persistent allergy to milk and egg [(48;49)]. The level of specific IgE-responses toward the DPYSP^{OH}S-containing domain could then provide additional information for the diagnosis and the management of peanut-allergic patients.

In conclusion, our study demonstrated the critical influence of post-translational modifications on the allergenic potency of Ara h 2. It also evidenced that short peptides encompassing the DPYSP^{OH}S-containing domain still constitute a potential risk for peanut-allergic patients. These results provide new insight into the allergenic activity of the most potent peanut allergen. Considering the diversity in the serology of peanut allergic patients in various parts of the world [(50)], it would be also interesting to determine whether the pattern of sensitization toward the DPYSP^{OH}S-containing domain and the conformational epitopes of Ara h 2 could be correlated to different methods of peanut processing and consumption.

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FIGURE LEGENDS

Fig. 1. Sequence comparison of the N-terminal part of Ara h 2.02 (Ara h 2.0201, UniProt accession number Q6PSU2), Ara h 2.01 (Ara h 2.0101, Q6PSU2-2), the deletion variant Ara h 2.Δ and Ara h 6 (Q647G9). Numbering of Ara h 6 is shown and hydroxyprolines in the DPYSP^{OH}S motifs are shown with shaded letters. Identical residues between Ara h 2 and Ara h 6 sequences are indicated with asterisks. The peptide (pep) 1-21 covered the N-terminal part of Ara h 2. The peptide containing two DPYSP^{OH}S motifs, thereby two hydroxyprolines (pep 2P^{OH}) corresponded to the domain found in Ara h 2.01 isoform. The peptide containing three DPYSP^{OH}S motifs, thereby three hydroxyprolines (pep 3P^{OH}) corresponded to the domain found in Ara h 2.02 isoform.

Fig. 2. Circular dichroism analysis shows a comparison of different isoforms of native and recombinant Ara h 2, the recombinant variant without DPYSP^{OH}S motif and r/a native and recombinant Ara h 2. *X-axis* shows the wavelength and *Y-axis* the molecular ellipticity.

Fig. 3. Impact of reduction and alkylation on the IgE-binding capacity of Ara h 2. Competitive inhibition binding of IgE antibodies from 18 peanut-allergic patients to native Ara h 2 was performed individually and 50% inhibitory concentration (IC50) was determined. For 9 out 18 tested sera, 50% inhibition of IgE-binding to Ara h 2 by r/a Ara h 2 was not reached at a concentration of 1 μM.

Fig. 4. Competitive inhibition of IgE-binding to native Ara h 2 for four representative sera. **A,** Comparison of the IgE-binding capacity of native and recombinant Ara h 2 and impact of reduction and alkylation. **B,** Influence of proline hydroxylation on the IgE-binding capacity of synthetic peptides overlapping the DPYSP^{OH}S-containing domain of Ara h 2. **C,**

Recapitulation of the IgE-binding capacity of Ara h 2.02 with an equimolar mixture of synthetic peptide pep $3P^{OH}$ and the deletion variant Ara h 2.Δ. The relative contribution of linear (purple) and conformational (orange) epitopes to the IgE-reactivity of Ara h 2, are estimated with the inhibitory capacity of pep $3P^{OH}$ and recAra h 2.Δ, respectively, and are shown for each patient. Sera 432, 841, 847 and 907 were diluted 1/200, 1/500, 1/300 and 1/500, respectively. Complementary data for 14 French patients and 5 American patients are shown in the Online Repository (Fig. E3 and E4).

Fig. 5. Mediator release assay with RBL SX-38 cells sensitized with immunopurified IgE antibodies from four peanut-allergic patients in response to increasing concentrations of different Ara h 2 variants and synthetic peptides. *X-axis* shows the concentration of the tested molecule and *Y-axis* the percentage of the reference release induced with anti-human IgE mAb LE27.

Figure No.1

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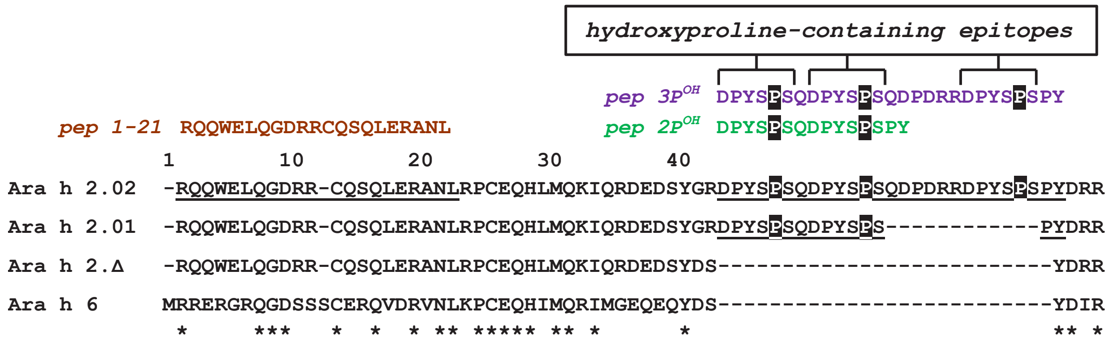

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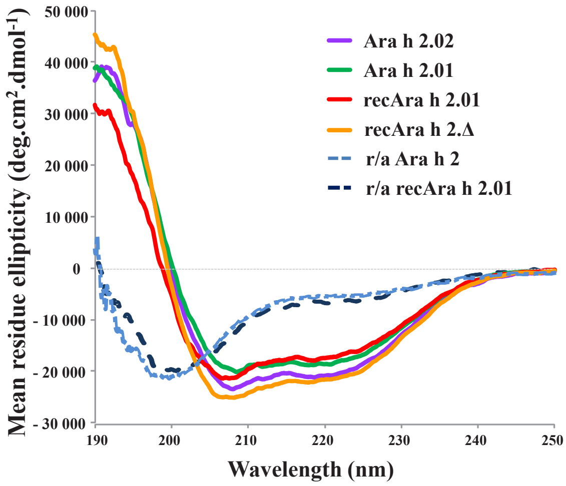

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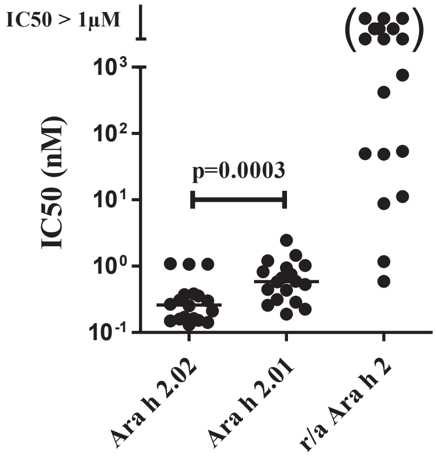

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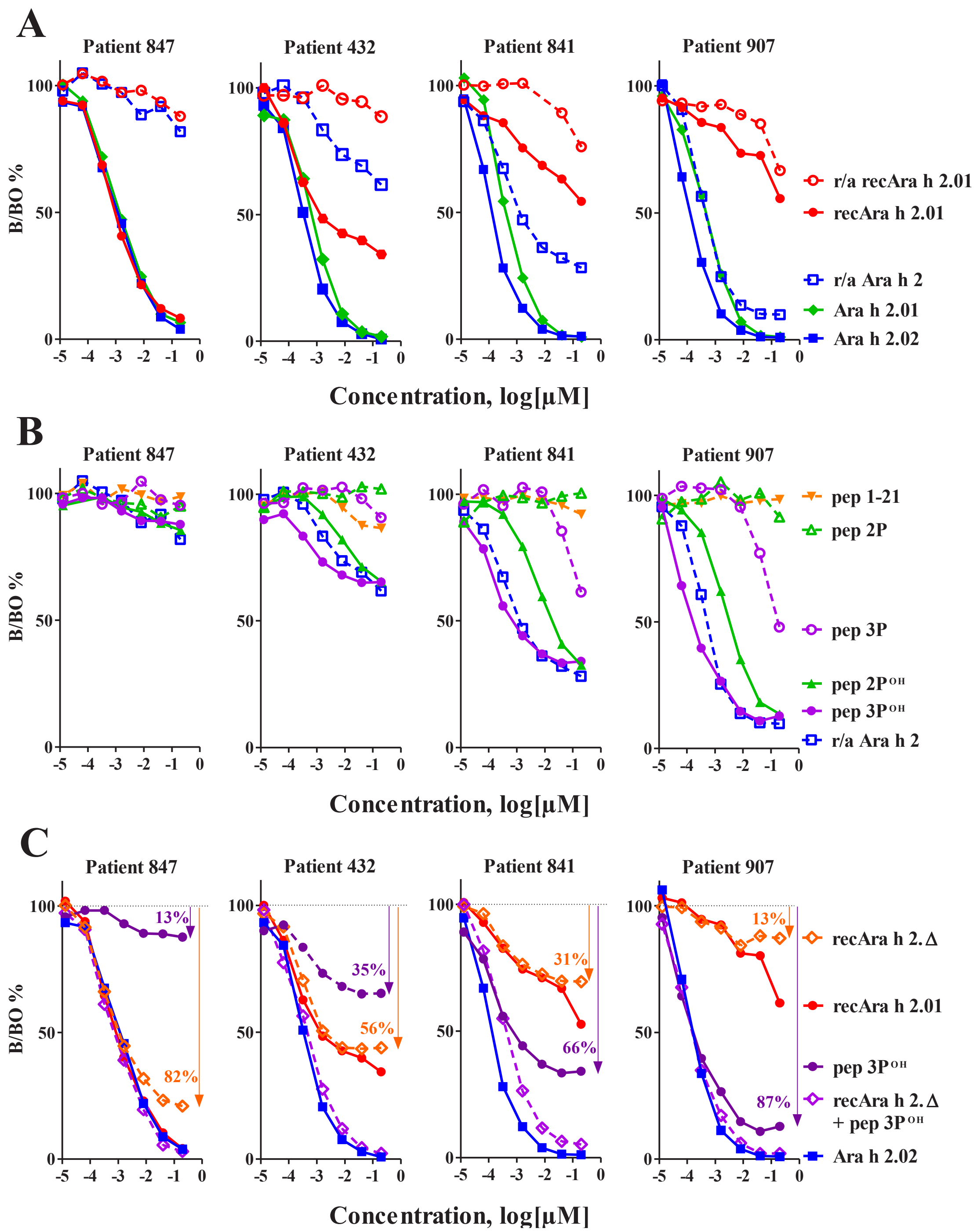

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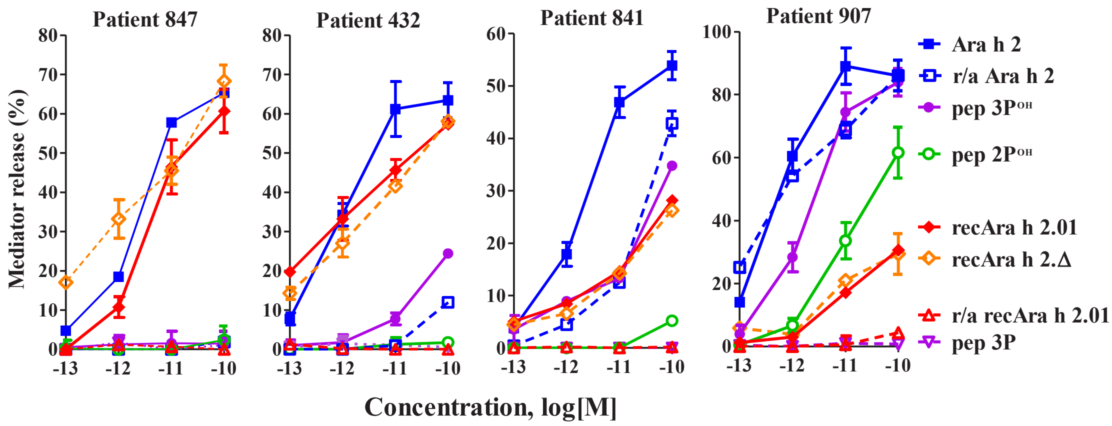

Online Repository Material

METHODS

Recombinant allergens: design, expression, refolding and purification.

The gene encoding Ara h 2.01 (Swiss-Prot accession number Q6PSU2-2, Fig. 1) was synthesized by using codons optimized for bacterial expression (Genscript USA Inc., Piscataway, NJ, USA) and inserted into the *E.coli* expression plasmid pET9c (Novagen-Merck, Damstadt, Germany). A 6xHis Tag and the HRV 3C protease cleavage site were added to the N-terminus of the recombinant allergen. The variant recAra h 2.Δ was generated by PCR-amplification of the expression plasmid without the sequence corresponding to the hydroxyproline-containing domain, with primers ATGAGTCGATCGTATGAGTCATAAGAGTCTTCATCACG and CGTATTCACCGTCGC and self-ligation of the PCR product restricted with PvuI enzyme.

Overnight cultures of transformed *E.coli* BL21 (DE3) were used to inoculate fresh medium at a dilution of 1:40. Cultures were grown at 37°C until the optical density at 600nm reached 0.5. The protein expression was induced by adding isopropylthio-β-galactoside (0.5mM) for 5 hours. After centrifugation, bacterial pellets were stored at -20°C until extraction. Frozen pellet corresponding to 1L of culture was resuspended in 100 ml of NaH_2PO_4/Na_2HPO_4 (50mM, pH 8)/NaCl (0.5M) buffer with protease inhibitors. After sonication and centrifugation (10 min, 5000g, 4°C), the pellet was resuspended in 100 ml of extraction buffer (NaH_2PO_4/Na_2HPO_4 50mM, pH 8, NaCl 0.5M, Urea 8M, DTT 5mM, Imidazole 20mM and proteases inhibitors) for 2h on rotary mixer at room temperature in order to solubilize inclusion bodies. His-tagged allergen was then purified with a HisTrap FF Crude column (GE Healthcare).

Refolding of recombinant protein was performed by direct dilution of the His-tagged purified fraction diluted at 0.2mg/ml in refolding buffer (Tris 0.5M, pH8, Glycerol 20%, L-Arginine 0.4M, GSH 2 mM and GSSG 2mM) and incubation overnight at 4°C with energetic shaking. After dialysis against Tris 100mM, the fraction of refolded allergen was purified by RP-HPLC as described previously[(1)]. Recombinant allergens were then characterised by gel electrophoresis, MALDI-TOF analysis and circular dichroism spectroscopy.

Purification of Ara h 2 isoforms

Whole peanut protein extract was prepared as previously described [(2)].Dialysed extract was fractionated by precipitation using ammonium sulphate, which was added to 40% saturation. After centrifugation, the pellet was discarded and the supernatant was dialysed against 20 mM phosphate pH 7.4 buffer. After addition of 0.5 M NaCl, the dialysate was submitted to affinity chromatography using Con A Sepharose. The flow-through fraction was dialysed against 20 mM Tris pH 7.4 buffer. 2S albumins were separated using a combination of preparative ion-exchange and reversed-phase chromatographies. Fractions containing isoforms of Ara h 2 were resuspended in buffer A (Urea 4 M, Tris 5 mM, pH 8.0) and further purified by anion exchange chromatography using a Source 30Q column (1.6*10cm) and an AKTA purifier system (GE healthcare, france). Isoforms were separately eluted using a 100 min linear gradient from 0 to 25% of buffer B(Urea 4 M, Tris 5 mM and 1 M NaCl pH 8.0).

Reduction and alkylation

Reduction of 2S-albumins was performed in urea 4M, EDTA 200µM and dithiotreitol 20mM during 2h at 56 °C. After cooling at room temperature, alkylation was performed by adding iodoacetamid (200mM), in the dark during 4 hours. After dialysis against potassium buffer (0.05M, pH 7.4), r/a 2S-albumins were characterised by gel electrophoresis, MALDI-TOF analysis and circular dichroism spectroscopy.

Peptide synthesis

Peptides were synthesized using a standard solid phase synthesis by the Fmoc (9-fluorenyl-methoxycarbonyl) continuous-flow method (peptide synthesizer 433A, Applied Biosystems, Foster City, CA). After standard procedure including TFA cleavage and ether precipitation, crude peptides were purified by RP-HPLC. The purified fraction was resuspended in potassium buffer (0.1M, pH 7.4) and peptides were characterised by MALDI-TOF analysis.

Mass spectrometry 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) operating at 20kV acceleration voltage, and equipped with a nitrogen UV laser (337 nm). Mass spectrometry analysis was performed on peptides or purified proteins mixed in a 1:1 ratio with a matrix solution of a-cyano-4-hydroxycinnamic acid or sinapinic acid. Analysis was performed in reflector or in a linear mode.

Circular Dichroism (CD) analysis

CD measurements were performed at 20°C on a JASCO-810 spectropolarimeter using 0.1 cm path length cells. A concentration of 0.1 mg/ml in 20mM phosphate buffer pH 7.4 was prepared for of the natural isoforms and recombinants of Ara h 2 samples. The spectra were recorded from 190 to 250 nm at a scanning speed of 100 nm/min with a 1s time constant, a 0.1 nm resolution and a 2 nm constant band pass. Three spectra were accumulated in each case. The averaged spectra were corrected by subtracting the baseline spectra obtained with the buffer alone under identical conditions. Mean residue weight ellipticities were calculated and expressed in units of degree*cm^2*dmol^{-1}.

Gel permeation chromatography

Gel Permeation Chromatography was performed to characterize the formation of peptide aggregates. Synthetic peptides were analyzed under physiological conditions at RT on a Stability GFC 50, 300*8 mm column (CIL, Cluzeau, France) coupled to an AKTA purifier system (GE Healthcare Life Sciences, France). A sample of peptide (100µL of 1 to 2 mg/ml) was applied to the column and eluted at 0.5 mL/min with phosphate buffer (150 mM KCl, 50 mM K_2HPO4/KH_2PO4) pH7.4. The eluate was monitored using UV absorbance at 220 nm. The column was calibrated using synthetic peptides with Molecular Weight of 1 and 6 kDa.

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Online Repository Figure Legends

Figure E1: Circular dichroism analysis shows a comparison of different synthetic peptides and the native Ara h 2.02. *X-axis* shows the wavelength and *Y-axis* the molecular ellipticity.

Figure E2: Analytical gel permeation chromatography of hydroxyproline-containing peptides under physiological conditions. Standard MW markers are shown across the top of the graph. *X-axis* shows the elution volume and *Y-axis* the absorbance at 220 nm.

Fig. E3. IgE-binding capacity of natural *vs* recombinant, native *vs* r/a Ara h 2 and of the hydroxylated peptide pep $3P^{OH}$. Competitive inhibition of IgE-binding to native Ara h 2 is shown for 18 sera from peanut-allergic patients [(3)]. The IgE-reactivity of r/a Ara h 2 increased concomitantly with the decrease of recAra h 2.01 IgE-reactivity.

Figure E4: A, Influence of proline hydroxylation on the IgE-binding capacity of synthetic peptides containing two or three $DPYSP^{OH}S$ motifs. Competitive inhibition of IgE-binding to native Ara h 2 for five sera from American peanut-allergic patients is shown. Of note, the IgE-binding capacity of r/a Ara h 2 was recapitulated with an equimolar mix of pep 1-21 and pep $3P^{OH}$ for patient D119; **B,** The IgE-binding capacity of Ara h 2.02 is recapitulated with an equimolar mixture of synthetic peptide pep $3P^{OH}$ and the deletion variant Ara h 2.Δ.

Fig. E5. IgE-binding capacity of native and r/a Ara h 6. Competitive inhibition of IgE-binding to native Ara h 6 for four representative sera is shown

Table E1. Clinical features and IgE responses of French peanut-allergic patients

Patients no.	Age/sex	Symptoms	Specific IgE levels to peanut proteins (IU/ml)				Total IgE (IU/mL)
			Ara h 1	Ara h 2	Ara h 3	Ara h 6	
101	9/M	U	168	237	321	239	532
102	11/M	QO, GU, V	164	158	279	197	796
109	8/F	A	325	446	538	529	2100
205	9/F	V, CP	5	6	8	12	806
222	6/M	GU, V	93	130	130	162	443
313	4/M	GU	111	133	163	138	5301
388	6/F	LO	204	174	341	235	1352
424	11/F	GU	81	64	95	90	325
432	4/F	LO, GU, V	74	87	108	106	361
441	9/M	LO, U, V	42	73	69	75	479
453	10/F	U	4	13	10	10	4523
486	7/M	A, R	41	65	96	78	412
572	7/F	GU	37	34	48	50	137
576	6/M	GU	13	30	33	28	114
841	6/M	LO, U, V	192	246	273	312	1283
847	5/M	LO, AS	176	221	220	159	697
907	9/F	LO, GU, V	555	667	916	935	1822
978	8/F	U, V	58	45	80	53	254

M, male; F, female; A, asthma; AO, angio-oedema; AS, anaphylactic shock; CP, cutaneous pruritus ; GU, generalized urticaria; LO, laryngeal oedema; QO, Quincke's oedema; R, rhinitis; U, urticaria; V, vomiting.

Table E2. Clinical features and IgE responses of American peanut-allergic patients

Patients no.	Age/sex	Symptoms	Specific IgE levels to peanut proteins (IU/ml)				
			WPPE	Ara h 1	Ara h 2	Ara h 3	Ara h 6
D80	13/M	V	156	107	95	122	112
D105	11/M	GU, AO, V	13.5	8.6	8.6	8.5	7.5
D114	28/F	GU, AO	24	15.9	10.6	20.2	9.5
D117	9/M	GU, AO, V	14.5	11.7	9.3	12	8.3
D119	15/M	GU, LO, A	64	45	40	51	55

M, male; F, female; A, asthma; AO, angio-oedema; GU, generalized urticaria; LO, laryngeal oedema; V, vomiting.

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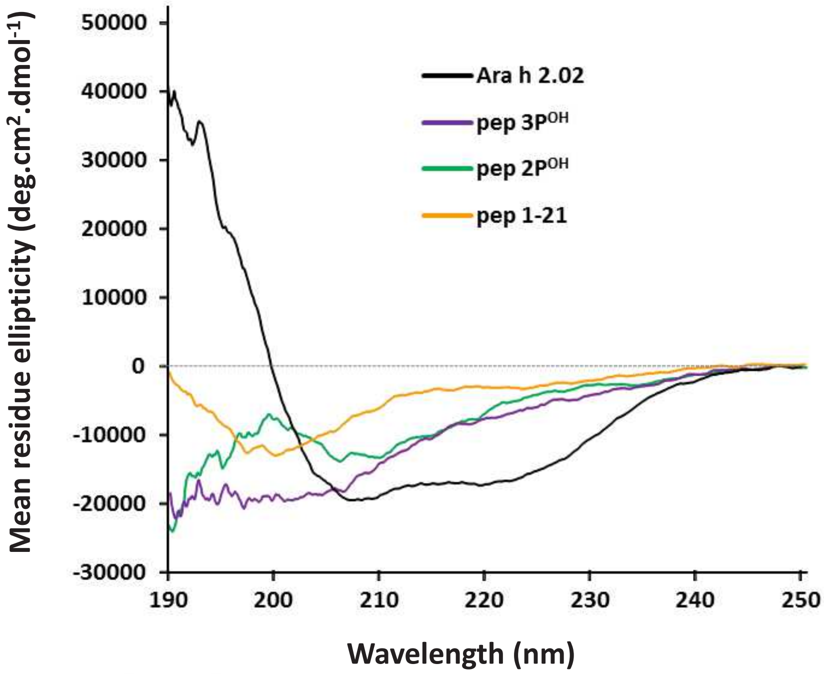

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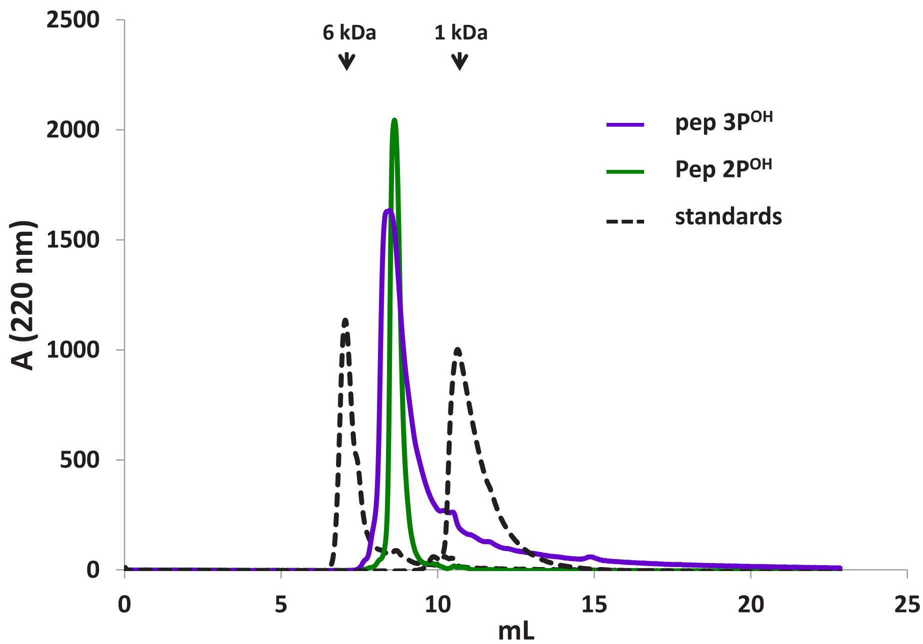

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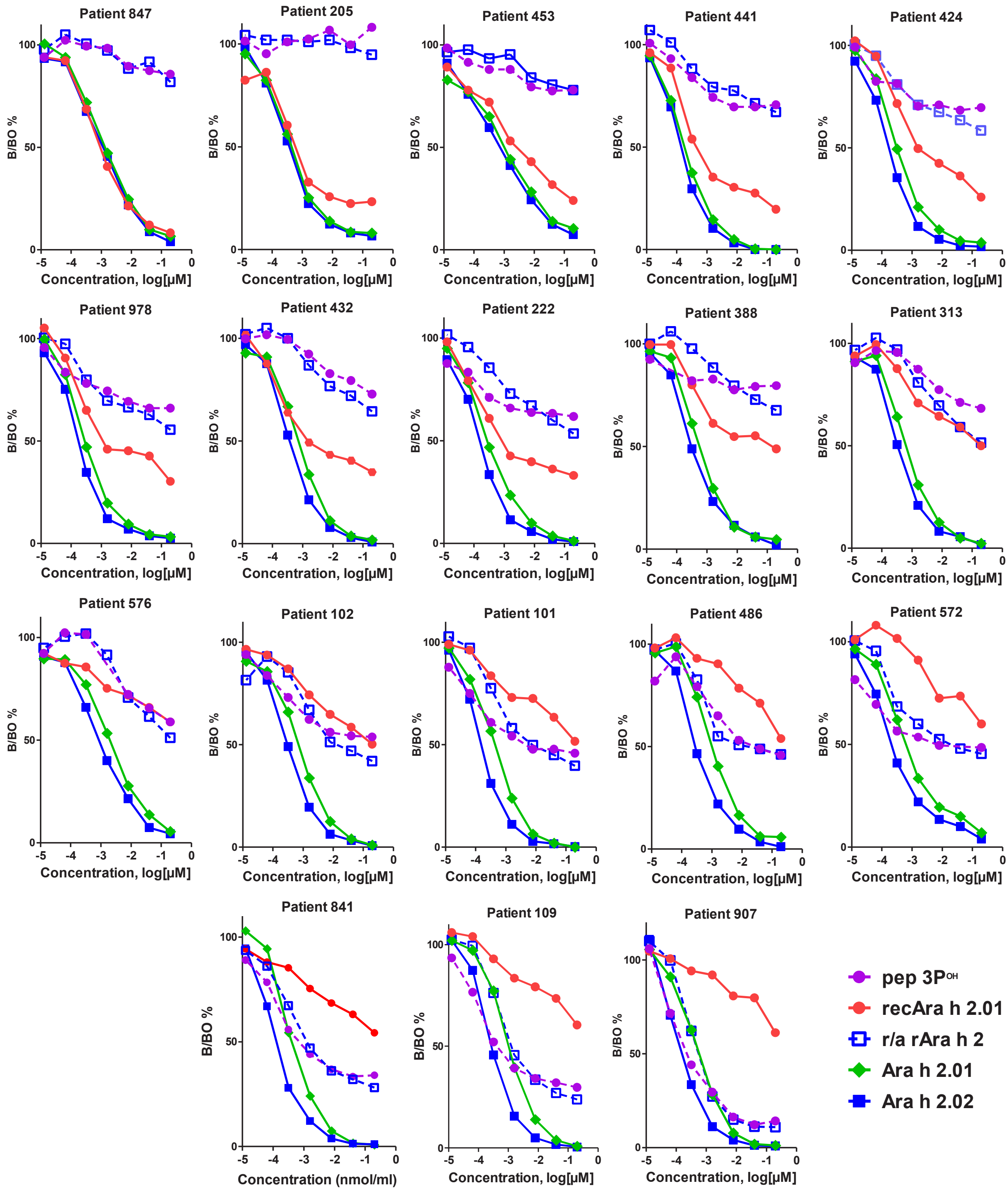

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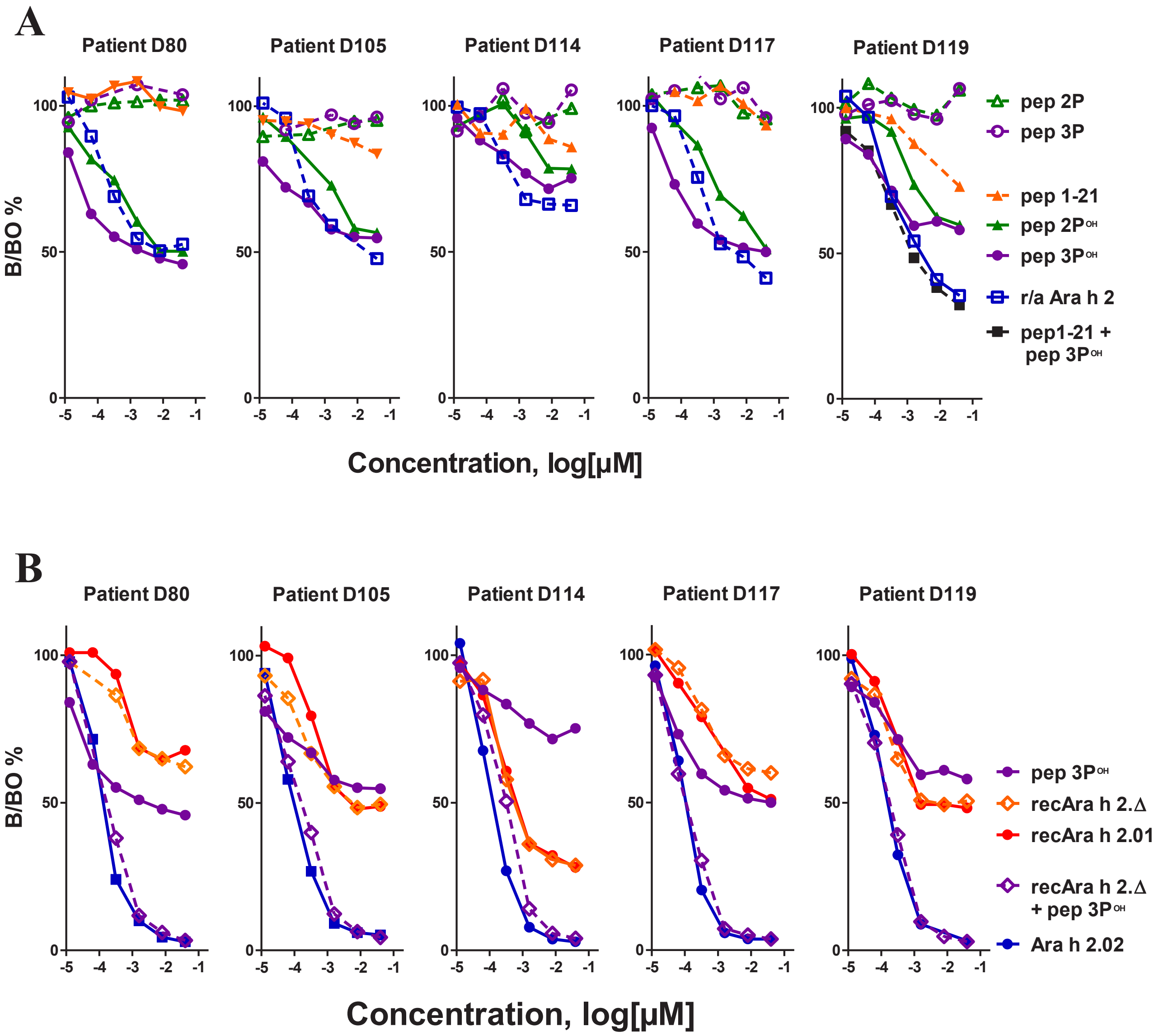

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