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

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1 **Conformational *versus* linear hydroxyproline-containing epitopes**
2 **of major peanut allergen Ara h 2**

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18

19 **Key messages:**

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

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

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

26 **Words count:** 3792

27

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

31

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

34

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

37

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41

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43 the National Institutes of Health; is on the American Board of Allergy and Immunology and
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45 Assessment (CISA) Network. The rest of the authors declare that they have no relevant
46 conflicts of interest.

47

48 **ABSTRACT**

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

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

54 **Methods:** Peptides containing DPYSP^{OH}S motifs were synthesized. A recombinant variant of
55 Ara h 2 without DPYSP^{OH}S motifs was generated by deletion mutagenesis. IgE reactivity of
56 18 French and 5 American peanut-allergic patients toward synthetic peptides and recombinant
57 allergens was assessed by IgE-binding inhibition assays and by degranulation tests of
58 humanized rat basophilic leukemia cells.

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

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

70

71

72

73 INTRODUCTION

74 Peanut is one of the most common causes of severe allergic reactions to food^(1;2). The
75 IgE-mediated peanut allergy affects more than 1% of the children and is outgrown by only
76 20% of the patients⁽³⁾.

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

84 Ara h 2 and Ara h 6 belong to the 2S-albumin family. They share a compact
85 conformation characterized by five α -helical structures and stabilized by a network of four
86 conserved disulfide bridges⁽²²⁾. This arrangement provides a core structure highly resistant to
87 proteolysis so that treatment of 2S-albumins with digestive enzymes does not affect
88 significantly their allergenicity⁽²³⁻²⁷⁾. Ara h 2 and Ara h 6 are 59% homologous but compared
89 to Ara h 6, two insertions of 14 and 26 amino acid residues occur in Ara h 2 major isoforms,
90 *i.e.* Ara h 2.01 and Ara h 2.02^(6;28). These insertions are exposed on a flexible surface loop
91 and comprise the repeated DPYSP^{OH}S motif, with the second proline being hydroxylated
92^(23;29). The DPYSP^{OH}S-containing domain has been reported to be a major linear IgE-binding
93 epitope^(30;31). The impact of proline hydroxylation on Ara h 2 IgE reactivity has not yet been
94 investigated, although this post-translational modification has been shown to influence the
95 IgE-binding to Phl p 1, a major allergen from timothy grass pollen⁽³²⁾. On the other hand,
96 conformation of 2S-albumins is also essential for the allergenic potency since suppression of
97 disulfide bridge formation in Ara h 2 and Ara h 6 by chemical reduction or by site-directed

98 mutagenesis reduced their IgE reactivity significantly^(26;30;33-35). In this regard, some studies
99 reported a predominance of IgE recognition of conformational epitopes on Ara h 2 while
100 others suggested a higher proportion of IgE-binding to linear epitopes^(30;33;36).

101 In the present work, we aimed to determine the relative contributions of linear and
102 conformational epitopes to the allergenic potency of Ara h 2. For this purpose, we first
103 characterized the IgE-reactivity of a stably unfolded Ara h 2 and of a properly refolded
104 recombinant Ara h 2. Discrepancies of IgE-binding capacity between the recombinant and
105 native allergens led us to investigate particularly the influence of proline hydroxylation. We
106 therefore compared the IgE reactivity of 18 French and 5 American peanut-allergic patients
107 toward native and recombinant Ara h 2 and toward synthetic peptides containing DPYSP^{OH}S
108 motifs with or without hydroxyproline. Moreover, considering that the DPYSP^{OH}S-containing
109 domain is located on a flexible surface loop in Ara h 2 and is absent in Ara h 6, this domain
110 was not expected to contribute significantly to the stability of the global fold of 2S-albumins
111^(23;37). We thus generated a recombinant variant of Ara h 2 lacking the DPYSP^{OH}S motifs in
112 order to investigate the contribution of conformational epitopes to the IgE-reactivity of Ara h
113 2 without any IgE-binding to the linear hydroxyproline-containing epitopes.

114

115 **METHODS**

116 **Human sera**

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

129

130 **Allergen preparations**

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

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

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

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

144

145 **Peptides**

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

159

160 **IgE-immunoreactivity analysis**

161 In agreement with the assumption from Albrecht *et al.* that “fluid-phase binding of IgE
162 antibodies is more relevant in relation to *in vivo* allergenicity” ⁽³⁰⁾, we developed a reverse
163 enzyme allergeo-sorbent test that is not based on the binding capacity of allergens immobilized
164 on solid phase but measures the binding of labelled allergens by patients’ IgE antibodies

165 captured by an anti-human IgE monoclonal antibody immobilized on the solid phase ⁽³⁹⁾. In
166 this test, plates were first coated with anti-human IgE monoclonal antibody LE27. Fifty
167 μL /well of serum from each patient at adequate dilutions were incubated overnight at 4°C .
168 After washing, $25\ \mu\text{L}$ of inhibitors (*i.e.* increasing concentrations of the tested molecule) and
169 $25\ \mu\text{L}$ of labelled native Ara h 2 were mixed and incubated for 4 h at room temperature.
170 Labelled Ara h 2 (all isoforms) used for this IgE-binding assay were prepared by covalent
171 linkage of the native protein to the tetrameric form of acetylcholinesterase (AChE) ⁽⁴⁰⁾.
172 Ellman's reagent was then used as AChE chromogenic substrate and absorbance at 414 nm
173 was measured ⁽⁴¹⁾. Results were expressed as B/B0. B0 and B represent the amount of labelled
174 Ara h 2 bound to immobilised IgE antibodies in the absence or presence of a known
175 concentration of inhibitor, respectively. The concentration inhibiting 50% of the IgE binding
176 to labelled allergen (IC50) was evaluated by using GraphPad Prism 5.01 (GraphPad Software,
177 Inc., La Jolla, CA, USA).

178

179 **Mediator release assay**

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

187

188 **Statistical analysis**

189 Data were analyzed using the non-parametric Wilcoxon matched pairs signed rank
190 test. Statistical analyses were performed with GraphPad Prism 5.01 software.

191 RESULTS

192

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

194 We first wanted to evaluate the loss of IgE-reactivity induced by chemical reduction of
195 the disulfide bridges and the resulting suppression of the conformational epitopes. Stable
196 unfolding of Ara h 2 was performed by reduction and alkylation (r/a). The unfolded state of
197 r/a Ara h 2 was confirmed by CD spectroscopy with a single spectrum minimum close to 200
198 nm instead of the two broad minima at 208 and 222 nm, typical for α -helical secondary
199 structures largely present in native Ara h 2 (Fig. 2). This denaturing treatment reduced
200 considerably the IgE-binding capacity of Ara h 2 (Fig. 3). However, for 9 of 18 patients (sera
201 313, 576, 101, 102, 486, 572, 841, 109 and 907), r/a Ara h 2 retained a significant IgE-
202 reactivity with an IC₅₀ ranging from 0.5 to 750 nM. Two patients (109 and 907) even
203 displayed an IgE response to r/a Ara h 2 comparable to that against the native allergen. Of
204 note, the isoform Ara h 2.02 displayed a slightly but significantly higher IgE-binding capacity
205 than Ara h 2.01 ($p=0.0003$). As illustrated in Fig. 4A, with four representative sera, and in
206 Fig. E3, the difference of IgE-reactivity between Ara h 2.01 and Ara h 2.02 increased
207 concomitantly with the IgE-binding capacity of r/a Ara h 2.

208

209 *IgE-binding capacity of native and recombinant Ara h 2*

210 We then wanted to assess whether a recombinant form of Ara h 2.01 shared similar
211 allergenic properties with its native counterpart. It thus appeared that recombinant and native
212 Ara h 2 displayed different patterns of IgE-reactivity among the patients (Fig. 4A and Fig.
213 E3). Interestingly, the IgE-reactivity of recAra h 2.01 was inversely proportional to that of r/a
214 Ara h 2. While native and recombinant Ara h 2 were similarly bound by IgE antibodies from
215 patient 847, r/a Ara h 2 was not recognized, thus indicating that most of Ara h 2-specific IgE

216 antibodies from patient 847 recognized conformational epitopes. In this regard, recAra h 2.01
217 also appeared to be properly refolded, as confirmed by CD analysis with the predominance of
218 α -helical structures (Fig. 2). Conversely, recAra h 2.01 was poorly recognized by IgE
219 antibodies from patient 907 whereas r/a Ara h 2 displayed an IgE reactivity almost as high as
220 that of the native allergen. In this case, most of Ara h 2-specific IgE antibodies from patient
221 907 recognized linear epitopes. Patients 432 and 841 displayed intermediate IgE-reactivity
222 toward recAra h 2.01 and r/a Ara h 2 (see also Fig. E3). It was then noteworthy that even
223 when recombinant and native Ara h 2 were reduced and alkylated, the IgE-binding capacity of
224 the recombinant allergen remained lower than that of the native form. The influence of post-
225 translational modifications that naturally occurs in peanut seeds but not in prokaryotes was
226 then further investigated.

227

228 ***Impact of proline hydroxylation on Ara h 2 IgE-reactivity***

229 Four peptides containing two or three DPYSP^{OH}S motifs, as found in Ara h 2.01 and
230 Ara h 2.02 respectively, were synthesized with or without hydroxyprolines (Fig. 1). As
231 illustrated by patients 841 and 907, peptides with hydroxyprolines displayed an IgE-binding
232 capacity at least 1000-fold higher than that of peptides without hydroxyprolines (Fig. 4B).
233 Inhibition of IgE-binding to Ara h 2 was always more efficient with the 27-AA-long peptide
234 3P^{OH}, with three DPYSP^{OH}S motifs, than with the 15-AA-long peptide 2P^{OH}, with only two
235 DPYSP^{OH}S motifs. Surprisingly, the IgE-binding capacity of the 27-AA-long peptide was as
236 high as that of the full-length r/a Ara h 2 for all the tested sera (Fig. 4B and Fig. E3), thus
237 suggesting that pep 3P^{OH} was bound by nearly all of the IgE antibodies recognizing linear
238 epitopes. The peptide 1-21 did not exhibit any significant IgE-binding capacity for any of the
239 French sera.

240

241 Evaluation of the relative contribution of linear vs conformational epitopes to Ara h 2**242 IgE-binding capacity**

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

249 IgE-binding to conformational epitopes could then be specifically inhibited with the
250 deletion variant recAra h 2.Δ and IgE-binding to linear epitopes could be inhibited with the
251 synthetic peptide pep 3P^{OH}. Accordingly, while IgE-binding to Ara h 2 was only partially
252 inhibited by pep 3P^{OH} or recAra h 2.Δ separately, an equimolar mixture of pep 3P^{OH} and
253 recAra h 2.Δ exhibited an inhibitory capacity similar to that of the native Ara h 2 (Fig. 4C).
254 The relative contribution of linear and conformational epitopes to the IgE-reactivity of Ara h
255 2 was then evaluated for each patient. For patient 847, around 82% of the IgE-binding to Ara
256 h 2 was due to the recognition of conformational epitopes while around 87% of the IgE-
257 binding to Ara h 2 was due to the recognition of linear epitopes for patient 907 (Fig. 4C). Five
258 sera from American peanut-allergic patients were similarly tested (Fig. E4). As observed with
259 French patients, hydroxylation of the synthetic peptides was required to obtain significant
260 IgE-binding to the synthetic peptides. IgE-binding to linear epitopes also appeared to be
261 restricted to the DPYSP^{OH}S-containing domain. Of note, pep 1-21 displayed a rather
262 significant IgE-binding capacity for one serum but still with a much lower affinity than pep
263 3P^{OH} (serum D119, Fig. E4A). American patients also displayed variable levels of
264 sensitization toward linear and conformational epitopes of Ara h 2 (Fig. E4B).

265

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

267 The capacity of the different variants and synthetic peptides from Ara h 2 to cross-link
268 IgE/FcεRI complexes was evaluated with a degranulation assay of RBL SX-38 cells. As
269 expected, when cells were passively sensitized with immunopurified IgE antibodies from
270 patient 847, the synthetic peptides did not display any allergenic activity and only properly
271 folded allergens, *i.e.* native and recombinant Ara h 2, were able to induce cell degranulation
272 (Fig. 5). In this case, we also confirmed that the synthetic peptides did not possess any
273 intrinsic ability to induce mediator release and that basophil degranulation was actually
274 dependent on the presence of specific IgE antibodies. Indeed, when cells were loaded with
275 IgE antibodies from patient 907, the peptides 3P^{OH} and, to a lesser extent, 2P^{OH} could trigger
276 cell degranulation almost as efficiently as the full-length allergen. Unfolded r/a Ara h 2 also
277 retained a strong allergenic potency. In contrast, the synthetic peptides without
278 hydroxyproline pep 3P and pep 2P (not shown) did not display any allergenic potency. The
279 pattern of cell degranulation induced with IgE antibodies from patient 432 and 841 were in
280 line with IgE-binding assays, since potency of the hydroxylated peptides correlated with that
281 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

307 domain 1-21 was reported to contain immunodominant IgE-binding epitopes⁽³⁰⁾, the
308 corresponding synthetic peptide rarely display a significant inhibitory capacity. The weak
309 IgE-binding capacity of r/a recAra h 2 also confirmed the absence of major linear epitope
310 when prolines are not post-translationally modified. This result is in agreement with Albrecht
311 *et al.* who reported that a mix of IgE-binding Ara h 2-derived peptide did not interfere
312 detectably with the IgE-binding to a recombinant form of Ara h 2⁽³⁰⁾. In contrast, Bublin *et al.*
313 observed that a considerable proportion of IgE binding to Ara h 2, Ara h 1 and Ara h 3 could
314 be inhibited with a mix of three synthetic peptides containing the N-terminal region of Ara h 2
315 and the repeated motif DPYSPS without hydroxylated proline⁽³⁶⁾. A weak IgE-reactivity of
316 the non-hydroxylated peptide pep 3P was also detected with patients 841 and 907. However,
317 the clinical relevance of this low affinity binding is questionable since pep 3P displayed no
318 allergenic potency when pep 3P^{OH} induced cell degranulation.

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

331 2P^{OH}, which is in agreement with previous studies suggesting a higher allergenicity of native
332 Ara h 2.02 compared to Ara h 2.01^(31;47).

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

348 Finally, the fact that the immunodominant linear IgE-binding epitopes of Ara h 2 are
349 almost exclusively located in the DPYSP^{OH}S-containing domain permitted us to use the
350 peptide pep 3P^{OH} and the recombinant variant Ara h 2.Δ to quantify the relative contribution
351 of linear and conformational epitopes to the IgE-binding. The level of sensitization to linear
352 and conformational epitopes appeared then to be quite variable among patients and we did not
353 evidence a predominant IgE-recognition of a certain type of epitopes over the other one. In
354 fact, when testing two different pools of sera from French or American peanut-allergic
355 patients, the contributions of linear and conformational epitopes to the IgE-binding capacity

356 of Ara h 2 were globally equivalent in both cases (data not shown). In this regard, comparison
357 of Ara h 2 IgE-reactivity between sera from French and American peanut-allergic patients did
358 not reveal any significant differences. The absence of proline hydroxylation in recombinant
359 Ara h 2 could then significantly affect the accuracy of component-resolved diagnostics for
360 most peanut-allergic patients by under-estimating the IgE response to Ara h 2. Recently, Lin
361 *et al.* developed a bioinformatics approach to identify patients with symptomatic peanut
362 allergy using peptide microarray immunoassay. The use of hydroxylated peptides as
363 biomarkers could also certainly increase the prediction performance. Moreover, sensitization
364 to linear epitopes has been associated with persistent allergy to milk and egg^(48;49). The level
365 of specific IgE-responses toward the DPYSP^{OH}S-containing domain could then provide
366 additional information for the diagnosis and the management of peanut-allergic patients.

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

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531
532

533 **FIGURE LEGENDS**

534 **Fig. 1.** Sequence comparison of the N-terminal part of Ara h 2.02 (Ara h 2.0201, UniProt
535 accession number Q6PSU2), Ara h 2.01 (Ara h 2.0101, Q6PSU2-2), the deletion variant Ara h
536 2.Δ and Ara h 6 (Q647G9). Numbering of Ara h 6 is shown and hydroxyprolines in the
537 DPYSP^{OH}S motifs are shown with shaded letters. Identical residues between Ara h 2 and Ara
538 h 6 sequences are indicated with asterisks. The peptide (pep) 1-21 covered the N-terminal part
539 of Ara h 2. The peptide containing two DPYSP^{OH}S motifs, thereby two hydroxyprolines (pep
540 2P^{OH}) corresponded to the domain found in Ara h 2.01 isoform. The peptide containing three
541 DPYSP^{OH}S motifs, thereby three hydroxyprolines (pep 3P^{OH}) corresponded to the domain
542 found in Ara h 2.02 isoform.

543

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

547

548 **Fig. 3.** Impact of reduction and alkylation on the IgE-binding capacity of Ara h 2.

549 Competitive inhibition binding of IgE antibodies from 18 peanut-allergic patients to native
550 Ara h 2 was performed individually and 50% inhibitory concentration (IC₅₀) was determined.
551 For 9 out 18 tested sera, 50% inhibition of IgE-binding to Ara h 2 by r/a Ara h 2 was not
552 reached at a concentration of 1 μM.

553

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

558 Recapitulation of the IgE-binding capacity of Ara h 2.02 with an equimolar mixture of
559 synthetic peptide pep 3P^{OH} and the deletion variant Ara h 2.Δ. The relative contribution of
560 linear (purple) and conformational (orange) epitopes to the IgE-reactivity of Ara h 2, are
561 estimated with the inhibitory capacity of pep 3P^{OH} and recAra h 2.Δ, respectively, and are
562 shown for each patient. Sera 432, 841, 847 and 907 were diluted 1/200, 1/500, 1/300 and
563 1/500, respectively. Complementary data for 14 French patients and 5 American patients are
564 shown in the Online Repository (Fig. E3 and E4).

565

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

Figure No.1

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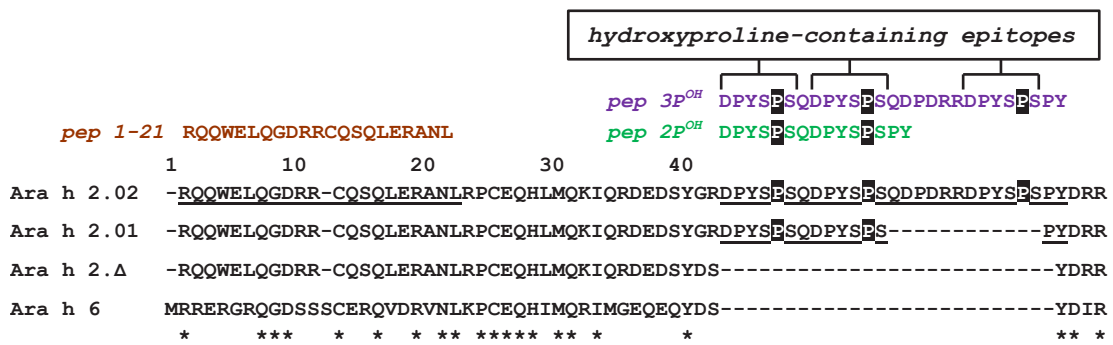


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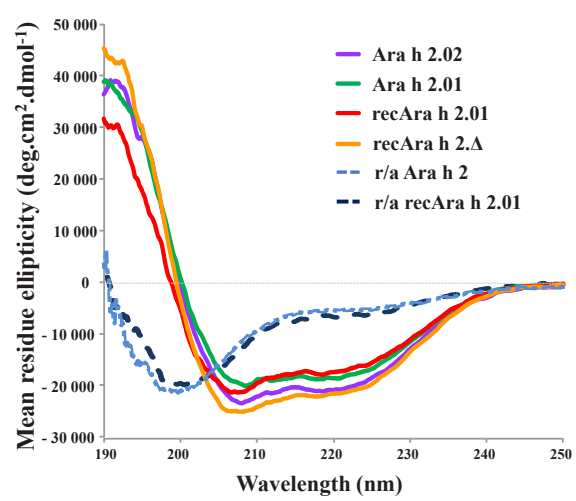


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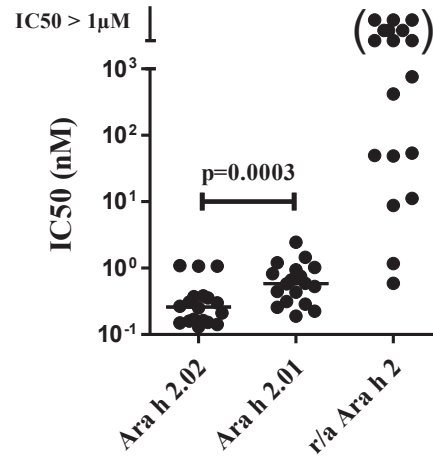


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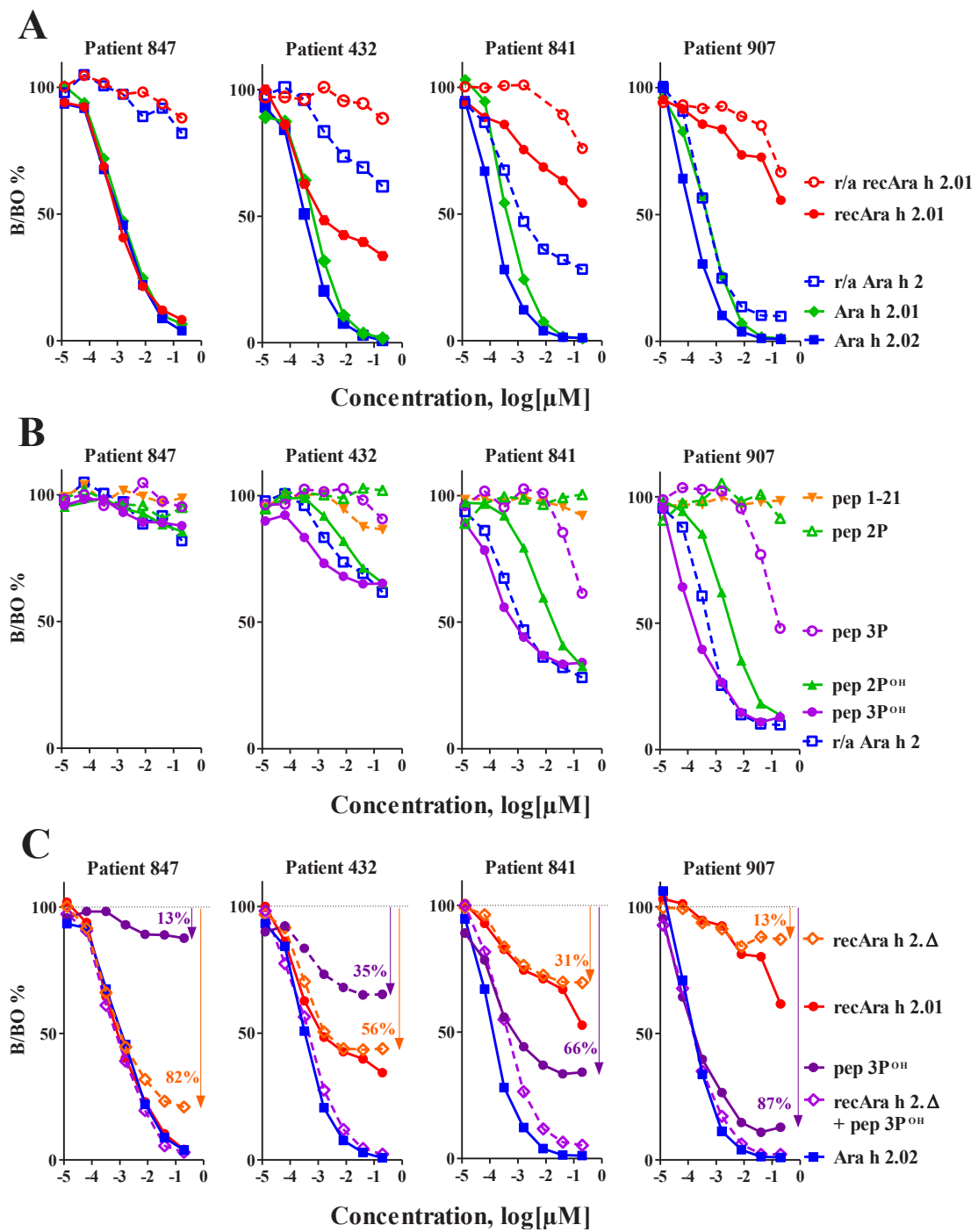
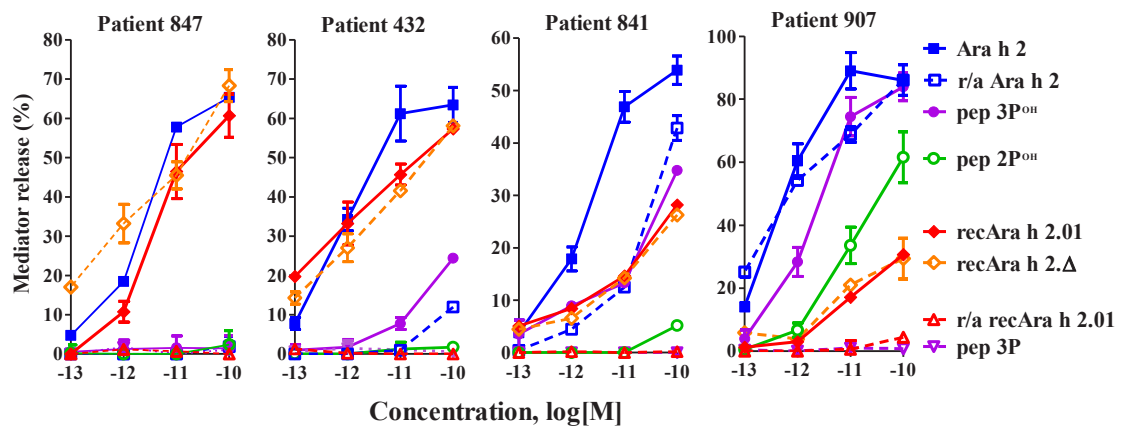


Figure No.5

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Online Repository Material

1

2

3 METHODS

4 **Recombinant allergens: design, expression, refolding and purification.**

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

13 Overnight cultures of transformed *E.coli* BL21 (DE3) were used to inoculate fresh
14 medium at a dilution of 1:40. Cultures were grown at 37°C until the optical density at 600nm
15 reached 0.5. The protein expression was induced by adding isopropylthio-β-galactoside (0.5mM)
16 for 5 hours. After centrifugation, bacterial pellets were stored at -20°C until extraction. Frozen
17 pellet corresponding to 1L of culture was resuspended in 100 ml of NaH₂PO₄/Na₂HPO₄ (50mM,
18 pH 8)/NaCl (0.5M) buffer with protease inhibitors. After sonication and centrifugation (10 min,
19 5000g, 4°C), the pellet was resuspended in 100 ml of extraction buffer (NaH₂PO₄/Na₂HPO₄
20 50mM, pH 8, NaCl 0.5M, Urea 8M, DTT 5mM, Imidazole 20mM and proteases inhibitors) for
21 2h on rotary mixer at room temperature in order to solubilize inclusion bodies. His-tagged
22 allergen was then purified with a HisTrap FF Crude column (GE Healthcare).

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

29

30 **Purification of Ara h 2 isoforms**

31 Whole peanut protein extract was prepared as previously described ⁽²⁾.Dialysed extract
32 was fractionated by precipitation using ammonium sulphate, which was added to 40% saturation.
33 After centrifugation, the pellet was discarded and the supernatant was dialysed against 20 mM
34 phosphate pH 7.4 buffer. After addition of 0.5 M NaCl, the dialysate was submitted to affinity
35 chromatography using Con A Sepharose. The flow-through fraction was dialysed against 20 mM
36 Tris pH 7.4 buffer. 2S albumins were separated using a combination of preparative ion-exchange
37 and reversed-phase chromatographies. Fractions containing isoforms of Ara h 2 were
38 resuspended in buffer A (Urea 4 M, Tris 5 mM, pH 8.0) and further purified by anion exchange
39 chromatography using a Source 30Q column (1.6*10cm) and an AKTA purifier system (GE
40 healthcare, france). Isoforms were separately eluted using a 100 min linear gradient from 0 to
41 25% of buffer B(Urea 4 M, Tris 5 mM and 1 M NaCl pH 8.0).

42

43 **Reduction and alkylation**

44 Reduction of 2S-albumins was performed in urea 4M, EDTA 200µM and dithiotreitol
45 20mM during 2h at 56 °C. After cooling at room temperature, alkylation was performed by
46 adding iodoacetamid (200mM), in the dark during 4 hours. After dialysis against potassium

47 buffer (0.05M, pH 7.4), r/a 2S-albumins were characterised by gel electrophoresis, MALDI-TOF
48 analysis and circular dichroism spectroscopy.

49

50 **Peptide synthesis**

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

56

57 **Mass spectrometry characterization**

58 Mass determination was carried out using a matrix-assisted laser desorption ionization-
59 time-of-flight instrument (MALDI-TOF, Voyager DE RP apparatus, PE Biosystems, France)
60 operating at 20kV acceleration voltage, and equipped with a nitrogen UV laser (337 nm). Mass
61 spectrometry analysis was performed on peptides or purified proteins mixed in a 1:1 ratio with a
62 matrix solution of α -cyano-4-hydroxycinnamic acid or sinapinic acid. Analysis was performed in
63 reflector or in a linear mode.

64

65 **Circular Dichroism (CD) analysis**

66 CD measurements were performed at 20°C on a JASCO-810 spectropolarimeter using 0.1
67 cm path length cells. A concentration of 0.1 mg/ml in 20mM phosphate buffer pH 7.4 was
68 prepared for of the natural isoforms and recombinants of Ara h 2 samples. The spectra were
69 recorded from 190 to 250 nm at a scanning speed of 100 nm/min with a 1s time constant, a 0.1
70 nm resolution and a 2 nm constant band pass. Three spectra were accumulated in each case. The

71 averaged spectra were corrected by subtracting the baseline spectra obtained with the buffer alone
72 under identical conditions. Mean residue weight ellipticities were calculated and expressed in
73 units of degree*cm²*dmol⁻¹.

74

75 **Gel permeation chromatography**

76 Gel Permeation Chromatography was performed to characterize the formation of peptide
77 aggregates. Synthetic peptides were analyzed under physiological conditions at RT on a Stability
78 GFC 50, 300*8 mm column (CIL, Cluzeau, France) coupled to an AKTA purifier system (GE
79 Healthcare Life Sciences, France). A sample of peptide (100μL of 1 to 2 mg/ml) was applied to
80 the column and eluted at 0.5 mL/min with phosphate buffer (150 mM KCl, 50 mM
81 K₂HPO₄/KH₂PO₄) pH7.4. The eluate was monitored using UV absorbance at 220 nm. The
82 column was calibrated using synthetic peptides with Molecular Weight of 1 and 6 kDa.

83

84 **Online Repository References**

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96

97

98 **Online Repository Figure Legends**

99

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

102

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

106

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

111

112 **Figure E4: A,** Influence of proline hydroxylation on the IgE-binding capacity of synthetic
113 peptides containing two or three DPYSP^{OH}S motifs. Competitive inhibition of IgE-binding to
114 native Ara h 2 for five sera from American peanut-allergic patients is shown. Of note, the IgE-
115 binding capacity of r/a Ara h 2 was recapitulated with an equimolar mix of pep 1-21 and pep
116 3P^{OH} for patient D119; **B,** The IgE-binding capacity of Ara h 2.02 is recapitulated with an
117 equimolar mixture of synthetic peptide pep 3P^{OH} and the deletion variant Ara h 2.Δ.

118

119 **Fig. E5.** IgE-binding capacity of native and r/a Ara h 6. Competitive inhibition of IgE-binding to
120 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.

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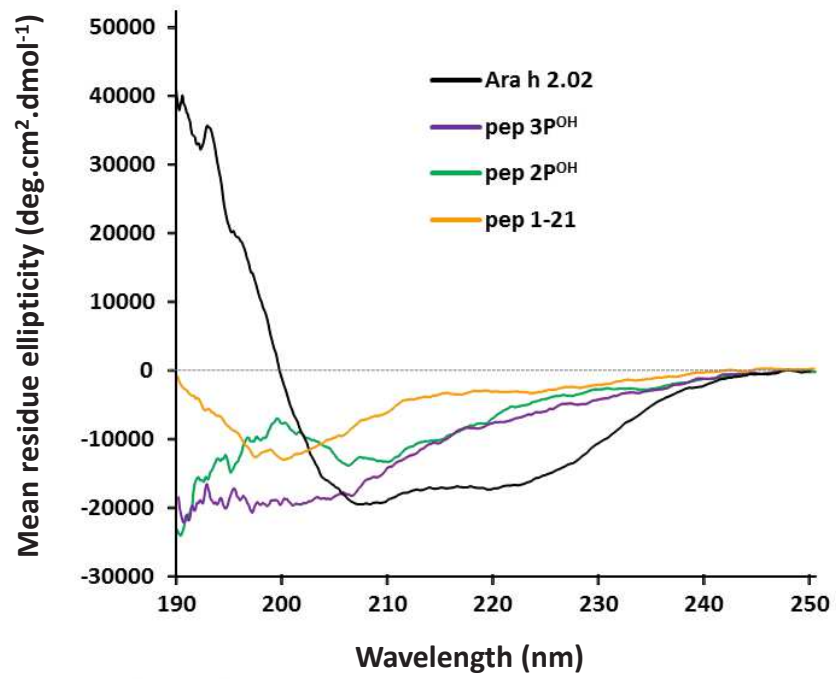
Table E2. Clinical features and IgE responses of American peanut-allergic patients

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

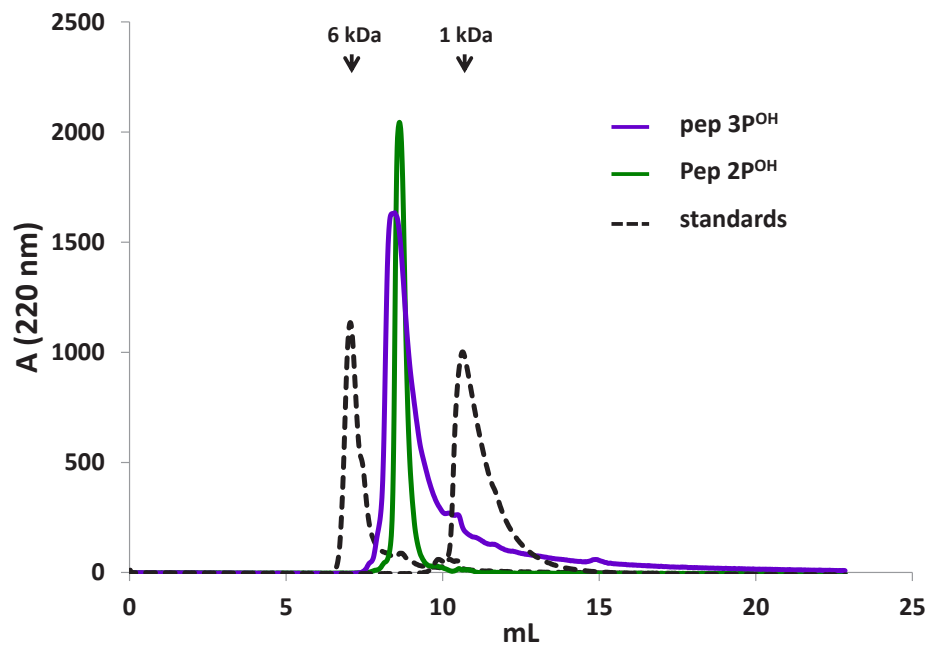
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M, male; F, female; A, asthma; AO, angio-oedema; GU, generalized urticaria; LO, laryngeal oedema; V, vomiting.

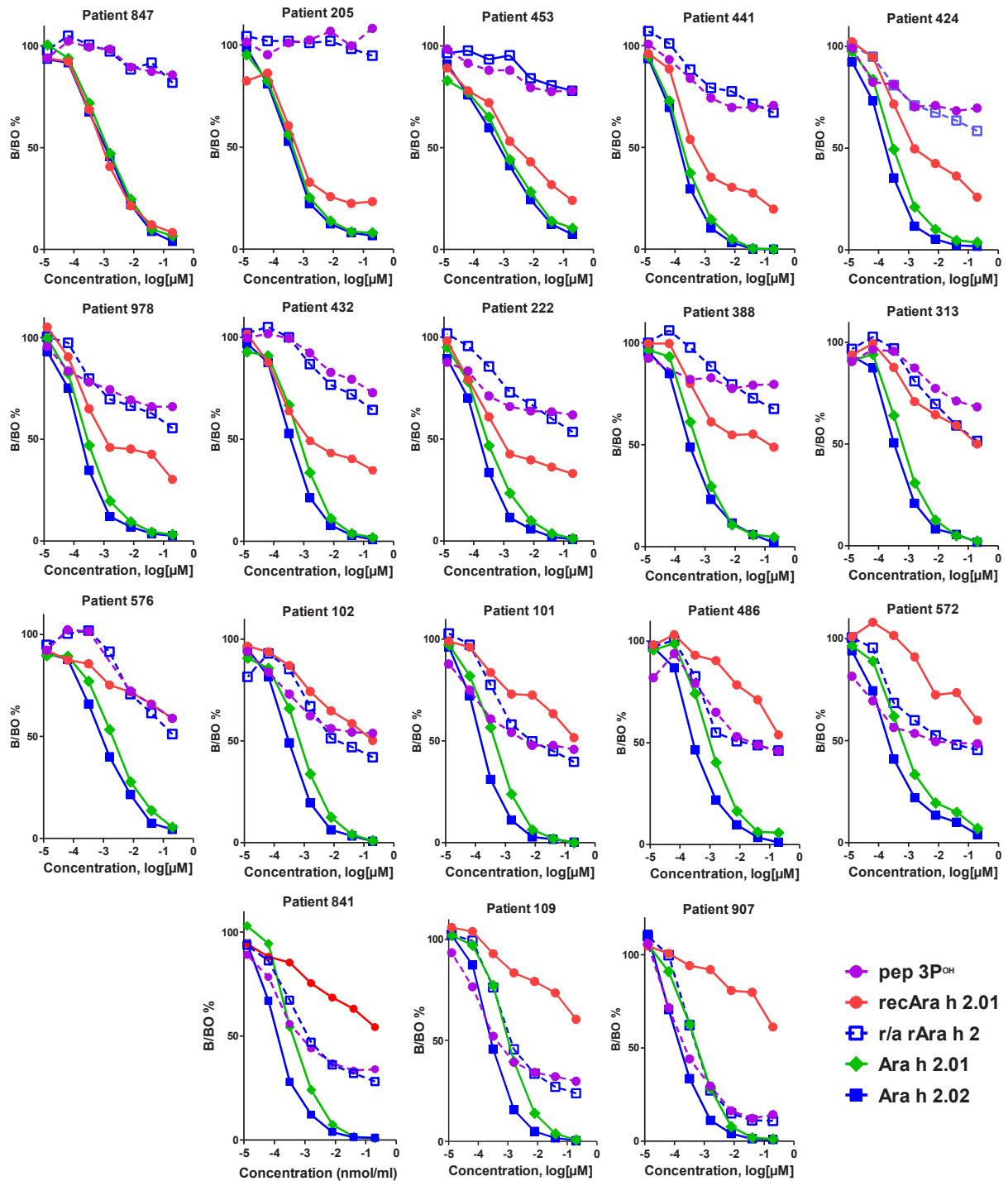
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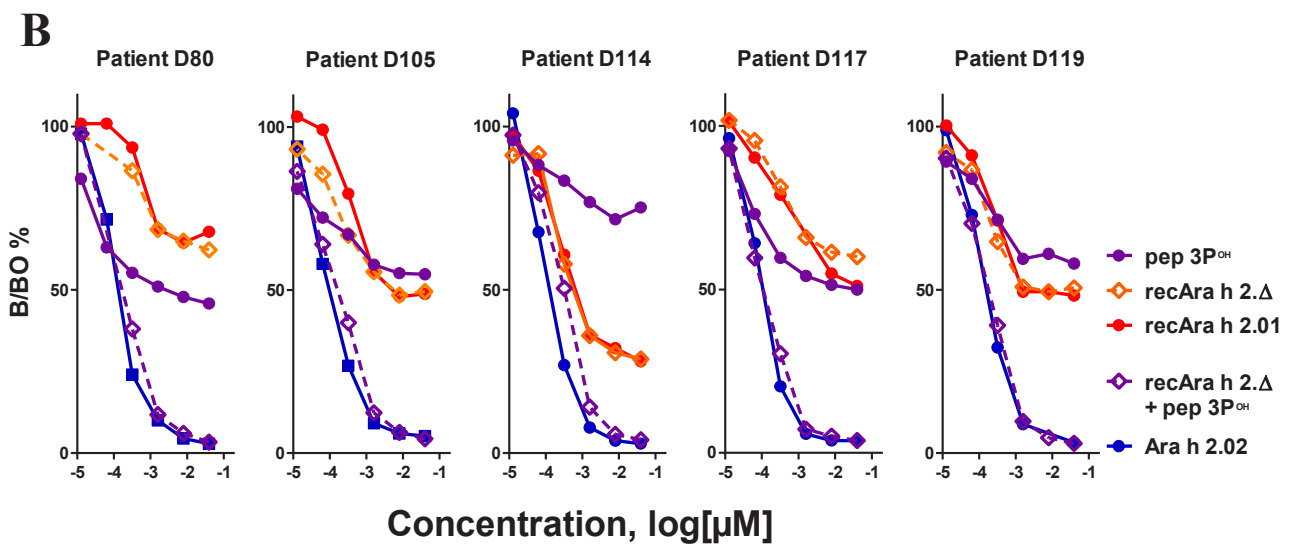
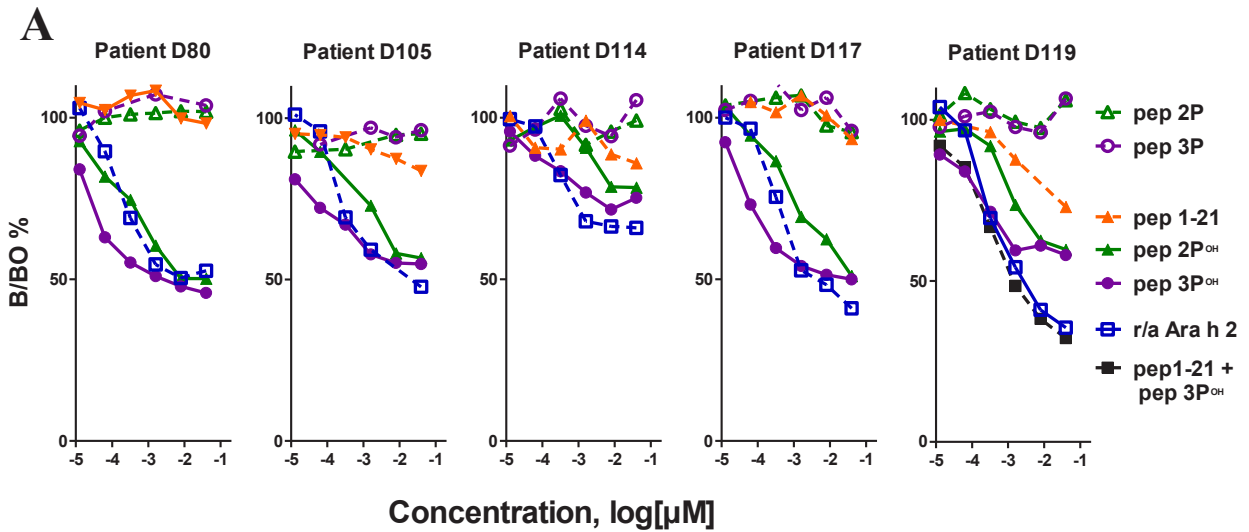


Repository E Figure No.2



Repository E Figure No.3





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