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11 **Identification of Pru du 6 as a potential marker allergen for almond** 12 **allergy**

13 **Short title: Molecular almond allergy diagnosis**

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Abstract

Background: Oral food challenges have demonstrated that diagnosis of almond allergy based on extract-sIgE tests display low specificity. Molecular allergy diagnosis is expected to improve accuracy, but its value in diagnosing almond allergy remains unknown.

Objective: To identify relevant almond allergens and examine their ability to improve almond allergy diagnosis.

Methods: IgE-reactive proteins were purified from almond kernels. IgE-binding to almond extract and the allergens was analyzed by quantitative ELISA using sera from 18 subjects with a proven almond allergy. The control group consisted of sera from 18 subjects allergic to peanut and/or tree nuts but tolerant to almond.

Results: Three IgE-binding proteins were identified: legumin (Pru du 6), alpha-hairpinin (Pru du 8) and mandelonitrile lyase (Pru du 10). Positive IgE (≥ 0.35 kU/L) to almond extract showed 94% sensitivity but only 33% specificity. IgE to Pru du 6 maintained high sensitivity (83%) and provided superior specificity (78%). Sera from almond-allergic subjects had significantly higher IgE levels to almond extract ($P < 0.0001$) and Pru du 6 ($P < 0.0001$) than sera from tolerant donors. Sensitization to Pru du 6 was highly specific for almond allergy, while frequencies of sensitization to legumins from peanut, walnut, hazelnut, and cashew were similar in both groups. IgE to Pru du 8 and Pru du 10 was less sensitive (41% and 67%), but showed specificities of 100% and 61%.

Conclusion: The use of almond allergens markedly increases the diagnostic specificity compared to the extract. Pru du 6 is a potential new molecular marker for almond allergy.

Keywords: almond allergens, molecular allergy diagnosis, diagnostic specificity, food allergy, tree nut allergy

56 Introduction

57 Almond along with walnut, hazelnut and cashew, is considered an important cause of tree nut allergy
58 in children and adults. Recent survey data in the U.S. reported almond and cashew with prevalences
59 of 0.7% each as the most common tree nut allergies in children and adults ^{1,2}. Australian studies
60 reported almond allergy prevalences of 0.2% in adolescents ³ and 0.3% in children ⁴. The proportion
61 of patients with peanut allergy who have concurrent almond sensitization is high with rates from 45%
62 to 71%, though only up to 11% reported allergic symptoms ⁵⁻⁷. Almond sensitization among those
63 with tree nut allergy was 54% and 44%, yet the prevalence of food-challenge-defined almond allergy
64 was $\leq 2\%$ ^{8,9}.

65 The main challenge in managing patients with suspected allergies to peanut and tree nuts is to
66 distinguish between concurrent allergy and asymptomatic co-sensitization due to multitude of food
67 sources to be avoided. Current data from oral food challenges (OFCs) have revealed that routine
68 almond allergy diagnosis based on clinical history confirmed by extract-based tests such as skin prick
69 test (SPT) and serum specific IgE (sIgE) test, is poor in predicting almond allergy ^{10,11}. From 590
70 patients who were referred for an OFC for suspected almond allergy 97% were sensitized to almond
71 extract, but only 5% failed almond challenge ¹⁰. Similarly, of 400 patients who underwent almond-
72 OFCs, 96% were sensitized to almond, yet 4% failed the challenge ¹¹. In both cohorts, neither almond
73 sensitization nor prior history of almond reaction were predictive of challenge failure. Consequently,
74 OFC remains the diagnostic standard for almond allergy to prevent unnecessary elimination diets.

75 In recent years, molecular allergy diagnosis using single allergens to quantify sIgE levels in serum has
76 been shown to be a valuable tool to elucidate distinct sensitization profiles and more accurately reflect
77 clinical reactivity, avoiding the need for a challenge. Allergens, such as Ara h 2 ¹², Jug r 1 ¹³, Cor a 14
78 ¹⁴, and Ana o 3 ¹⁵ were described as superior in predicting allergic reactions to peanut, walnut,
79 hazelnut and cashew, compared to the extracts. In contrast to peanut and other tree nuts, the value of
80 almond allergens in diagnosing almond allergy is unknown. Prior to this study, there were five
81 almond allergens registered in the WHO/IUIS database of allergens: Pru du 3 (non-specific lipid-
82 transfer protein) ¹⁶, Pru du 4 (profilin) ¹⁷, Pru du 5 (60S ribosomal protein) ¹⁸, Pru du 6 (legumin) ^{19,20},
83 and Pru du 8 (α -hairpinin antimicrobial protein) ²¹. Pru du 6, also known as amandin, is a highly

84 abundant storage protein which is frequently recognized by IgE from almond sensitized patients ¹⁹.
85 Almond profilin Pru du 4 seems to be relevant for patients with grass pollinosis ¹⁷. The other three
86 allergens have been identified by cDNA cloning from immature almonds but their presence in mature
87 almond kernels was not investigated ^{18,22}.

88 The aim of this study was to i) identify and purify IgE-binding proteins from almond kernels, ii)
89 establish the allergen sensitization profiles of patients with proven almond allergy and iii) investigate
90 the ability of individual almond allergens to discriminate between almond-allergic and almond-
91 tolerant patients with peanut and other tree nuts allergies.

92 **Materials and Methods**

93 A more detailed version of the Methods section can be found in the supplement.

94 **Patient's sera**

95 Eighteen patients with almond allergy were included in the study: 14 sera were collected from
96 patients with a positive double-blind, placebo-controlled food challenge (DBPCFC) to almond and 4
97 from patients with a convincing history of almond allergy. DBPCFCs were performed at the Sean N.
98 Parker Center for Allergy and Asthma Research at Stanford University using standardized
99 methodology according to validated guidelines ^{23–25} and as previously described ²⁶. The inclusion
100 criteria for DBPCFC were suspected almond allergy, defined as an sIgE ≥ 0.35 kU/L and/or a positive
101 skin prick test (SPT) to almond. The blood was drawn before the challenge. A challenge was
102 considered positive if objective symptoms occurred within 4 hours of exposure. Inclusion criteria for
103 four almond allergic subjects enrolled at the Department of Dermatology, University Hospital St.
104 Poelten were a history of an allergic reaction after ingestion of almond and sIgE or positive SPT to
105 almond.

106 To compare allergen recognition profiles between almond allergic and almond tolerant patients and to
107 analyse the diagnostic specificity of the almond allergens, a control group consisting of 18 subjects
108 was included. In the tolerant group, peanut and/or nut allergic patients were included who reported
109 regular consumption of almonds without any allergic symptoms. The inclusion criteria were a
110 clinically proven history of a peanut and/or tree nut allergy but tolerance of almond and sIgE to
111 peanut and/or tree nuts (Tables 1B and S1). An additional negative control group consisted of 4 atopic
112 subjects with no history of food allergy and negative sIgE tests to food allergens. Allergen-sIgE levels
113 were measured by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) or ALEX (Macro Array
114 Diagnostics GmbH, Austria). The use of clinical data and serum samples for this study was approved
115 by the local ethics committees (Stanford Institutional Review Board, the Ethics Committee of Lower
116 Austria, and the Ethics Committee of the Medical University of Vienna) and signed informed consent
117 was obtained from all patients.

118 **Purification and characterization of almond allergens**

119 IgE-binding proteins were purified from almonds using different protein extraction conditions and
120 diverse chromatographic techniques. Protein identification was performed by Nano-LC ESI-Orbitrap
121 MS/MS and N-terminal sequencing. Purity of the proteins was analyzed by SDS-PAGE, 2D
122 electrophoresis and Coomassie Brilliant Blue staining. Secondary structure content was determined by
123 CD-spectroscopy. To demonstrate IgE-binding, immunoblotting and quantitative ELISA were
124 performed. The detailed protocols are described in the supplement.

125 **Statistical analysis**

126 The Mann-Whitney U-test was used for comparing allergen-specific IgE levels of patients with and
127 without almond allergy. Chi-Square test was applied to assess statistically significant differences
128 between the percentages of sera from the two groups reacting with individual allergens or extracts.
129 Receiver operating characteristics (ROC) curves were generated to evaluate the diagnostic capacity of
130 the test for specific IgE levels. An optimal cutoff point for sIgE to the tested allergens was obtained
131 using the Youden index (sensitivity + specificity - 1). Paired correlation was calculated by using the
132 Pearson test. P-values <0.05 were considered significant.

133 **Results**

134 **Patients' characteristics**

135 Sera from 18 patients with clinically relevant allergic reactions following ingestion of almonds were
136 included in this study (Table 1A). The most common symptoms elicited by almond were urticaria
137 (n=10), rash (n=4), and angioedema (n=6). Most of the patients (56%) had a history of immediate
138 reaction to peanut, 44% to cashew, 39% to hazelnut and 44% to walnut. As control group, 18 sera
139 from patients with allergy to at least one tree nut or peanut were used (Table 1B). At the time of
140 inclusion, all control patients indicated to consume almond regularly without allergic symptoms.
141 Similar to the almond-allergic group, most of the patients had peanut (72%) and hazelnut allergy
142 (67%) followed by walnut allergy (61%). The total IgE levels and sIgE levels to peanut, hazelnut,
143 walnut, Brazil nut and macadamia did not differ significantly between the two groups (Table S1).

144 **Identification of novel almond allergens**

145 SDS-PAGE analysis of almond extract showed multiple protein bands in the range of 10-100 kDa
146 (Figure 1, lanes 1-2). Immunoblotting of the extract with a pool of sera from almond allergic patients
147 (Table 1A, sera 4, 5, 10 and 16) showed several IgE-binding signals in this range (Figure 2A, lanes 1-
148 2). After purification, IgE-binding proteins were identified as Pru du 6, Pru du 8, and mandelonitrile
149 lyase 2, a novel plant food allergen (Figure 1, lanes 3-8 and Table S2). The complete inhibition of
150 IgE-binding after pre-incubation of the serum pool with the mixture of the three proteins indicated
151 that the IgE-binding bands in the extracts represent the isolated proteins (Figure 2A, lane 3). Pru du 6
152 migrates as a band of around 60 kDa and dissociates under reducing conditions into two subunits of
153 20 and 40 kDa (Figure 1, lanes 3-4). As shown in 2D-PAGE (Figure S1), Pru du 6 occurs in different
154 processed forms indicated by multiple spots of similar molecular masses but varying isoelectric points
155 (pIs). Pru du 8 migrates at 13 kDa under reducing and at 26 kDa under non-reducing conditions
156 (Figure 1, lanes 5-6 and Figure S1) indicating dimer formation. The newly identified mandelonitrile
157 lyase 2 migrates at about 60 kDa and a pI of 5.5 (Figure 1, lanes 7-8 and Figure S1). This allergen was
158 designated Pru du 10 by the WHO/IUIS Allergen Nomenclature Sub-Committee.

159 The three proteins showed high purity (> 95%) and no cross-contamination with other allergens, as
160 determined by high-accurate tandem mass spectrometry (Table S2). A clear IgE-reactivity to Pru du 6
161 and Pru du 10 was observed under non-reducing (Figure 2A, lines 5-6 and 11-12) as well as reducing
162 conditions (Figure 2B, lines 1 and 5). However, IgE-reactivity to Pru du 8 was only observed under
163 non-reducing conditions (Figure 2 A, lines 8 and 3). No considerable reduction of IgE-binding to Pru
164 du 10, a glycoprotein, was observed after inhibition of IgE antibodies specific to CCDs using HRP,
165 indicating involvement of protein residues in the IgE-binding (Figure 2A, lines 11-12). Similarly, IgE-
166 binding to the other allergens was not altered upon pre-incubation of sera with HRP (Figure 2A and
167 2B).

168 Changing protein extraction conditions such as the use of different buffers, pH values, salt
169 concentration and water-alcohol ratios did not result in detection of other IgE-binding proteins (e.g.
170 vicilins and 2S albumins), typically present in other tree nuts.

171 **Sensitization profiles to individual almond allergens differ between almond-allergic and** 172 **almond-tolerant subjects**

173 IgE reactivity to almond allergens differed substantially between the almond-allergic and almond-
174 tolerant group (Table 1, Figure 3 and Table S1). Almond-allergic patients had significantly more
175 frequently ($P<0.001$) IgE specific to Pru du 6 than patients who tolerate almond. Sixteen of 18 (89%)
176 sera from almond-allergic subjects had specific IgE levels of ≥ 0.35 kU_A/L, while only 22% (4 of 18)
177 of sera of the control group were positive to the allergen. Rates of IgE-binding to other allergenic
178 legumins from peanut (Ara h 3), walnut (Jug r 4), hazelnut (Cor a 9), and cashew (Ana o 2) were
179 similar among almond-allergic and asymptomatic patients indicating that IgE-binding to Pru du 6 was
180 specific for almond allergy (Figure 4).

181 Sensitization to Pru du 8 was observed in 44% of the almond-allergic patients and to the newly
182 identified allergen Pru du 10 in 67%. Only one serum from the control group reacted with Pru du 8,
183 and 7 sera with Pru du 10. Sixteen almond-allergic patients (89%) were sensitized to at least one
184 almond allergen and six (33%) to all three allergens. The two sera negative to the three almond
185 allergens were positive to Bet v 1 and the Bet v 1-homologous allergens Cor a 1.04, Mal d 1 and Gly
186 m 4. One of these patients also had IgE specific to Pru p 3, as a representative of almond nsLTP

(Table 1). The IgE-binding frequency to Bet v 1 was 67% and in the control group 72%. Sensitization to Phl p 12, as a representative of almond profilin, was observed in 28% of almond allergic and 6% of almond tolerant subjects. No serum from the atopic control subjects reacted with almond allergens. There was a strong correlation ($R^2 = 0.9$) between the IgE reactivity to almond extract and sIgE- reactivity to the three almond allergens, expressed as the sum of sIgE values of the three individual allergens (Figure S2), indicating that IgE to these allergens represents the majority of almond-specific IgE. When specific IgE antibody levels to individual allergens were investigated, sera from almond-allergic subjects had significantly higher IgE specific to Pru du 6 (median, 8.7 kU_A/L; range, 1.1-181.7, $P < 0.0001$), Pru du 8 (median, 7.4 kU_A/L; range, 0.5-159.0 kU_A/L, $P = 0.013$) and Pru du 10 (median, 3.6 kU_A/L; range, 0.4-95.1, $P = 0.031$) compared to the tolerant group (Figure 3 and Table S1). The median levels of sIgE to Bet v 1 and Pru p 3 were similar between reactive and non-reactive patients. IgE specific to cashew extract (median, 17.3 kU_A/L, $P = 0.0030$) and Ana o 3 (median, 20.9 kU_A/L; $P = 0.0073$) was significantly higher in the group of allergic patients owing to the fact that most patients had co-existing cashew allergy. There was no difference in the quantity of IgE specific to other extracts or allergens listed in Table S1.

Using single almond allergens increases diagnostic specificity

Seventeen (94%) of 18 patients with almond allergy and 12 (67%) of 18 almond-tolerant patients had sIgE ≥ 0.35 kU_A/L to almond extract. The median level of sIgE to almond extract (12.6 kU_A/L; range, 0.4-317.2 kU_A/L, $P < 0.0001$) was significantly higher in sera from patients allergic to almond compared with tolerant patients (Table 1 and Table S1). Positive sIgE to almond extract was highly sensitive (94%), but not specific (33%). At the same threshold level, Pru du 6 maintained high sensitivity (83%), but provided superior specificity (78%). Pru du 8 and Pru du 10 were less sensitive (44% and 67%), but with specificities of 100% and 61%. The cut-off almond-sIgE level that predicted clinical reactivity with the highest sum of sensitivity and specificity was 5.2 kU_A/L, with a sensitivity of 72% and a specificity of 100% (Table 2). The optimal cutoff point of IgE to Pru du 6 was 1.8 kU_A/L, with sensitivity and specificity of 77% and 94%, respectively. The calculated optimal cut-off point for Pru du 8 was 0.35 kU_A/L with a sensitivity of 44% and specificity of 100%, and for Pru du 10 0.75 kU_A/L with a sensitivity of 61% and specificity of 78% (Table 2). Based on the calculations

216 of areas under the ROC curves, sIgE to almond extract and Pru du 6 had the highest accuracy in
217 discriminating between allergic and tolerant individuals: area (95% CI), almond extract (0.89), Pru du
218 6 (0.87), Pru du 10 (0.71), and Pru du 8 (0.70) (Figure 5).

219 Discussion

220 Recent studies have demonstrated that diagnosis of almond allergy by extract-based tests poorly
221 differentiate between sensitized and allergic individuals. Thus, the diagnosis is heavily reliant on
222 OFCs. Molecular diagnostics, which measure allergen-specific IgE, offer the potential to improve the
223 diagnostic accuracy, but no information about diagnostic performance of almond allergens is
224 available. In this study, we identified relevant allergens in almond kernels and determined allergen
225 sensitization profiles of almond-allergic and almond-tolerant patients. Furthermore, we evaluated for
226 the first time the diagnostic value of molecular diagnosis of almond allergy in particular with regard
227 to specificity compared with that of an extract-based diagnosis.

228 Our results showed a difference in the allergen compositions of almond compared with peanut and
229 other tree nuts. Allergies to peanut and tree nuts are typically associated with sensitization to seed
230 storage proteins (2S albumins, legumins and vicilins) and additionally often to one or several less
231 prominent allergens. With exception of almond legumin (Pru du 6), which was also previously
232 detected as an important allergen ¹⁹, 2S albumin and vicilin, typically abundant in other nuts, could
233 not be detected in almond kernels. Although recently a vicilin-related cDNA was isolated from
234 immature almonds and a recombinant protein produced has been shown to bind IgE, the presence of
235 its natural counterpart in mature almonds was not confirmed ²². In line with our results, neither a
236 vicilin nor a 2S albumin was identified by mass spectrometric proteome analysis of the almond kernel
237 ²⁷.

238 Instead, we purified two almond-specific allergens, Pru du 10 and a 13 kDa IgE-binding protein
239 which was identified as a C-terminal fragment of previously reported almond allergen Pru du 8.
240 Recombinant Pru du 8 obtained by cDNA cloning from immature almonds is a 26 kDa protein
241 belonging to the α -hairpinin family ²⁸. Our results indicate that this sequence codes for a
242 preproprotein which in mature almond undergoes post-translational proteolytic processing, as has
243 been shown for a homologous protein from wheat ²⁸. Interestingly, IgE-binding peptides from the α -
244 hairpinin protein family have also been identified in peanut ²⁹ and walnut ³⁰, but in contrast to almond
245 those peptides originate from the N-terminal region of vicilin preproproteins.

246 Another interesting finding of this study is the identification of almond mandelonitrile lyase 2 as a
247 novel allergen, which was designated Pru du 10. Mandelonitrile lyase 2, a key enzyme for a process
248 known as cyanogenesis is the most studied almond protein. It is a flavoprotein belonging to the
249 glucose-methanol-choline oxidoreductase family. Although N-glycosylation sites were previously
250 identified by crystallography ³¹, our results showed that glycan moieties are not involved in IgE
251 reactivity to Pru du 10.

252 As a next step, we used the panel of purified almond allergens to establish sensitization profiles of 18
253 almond allergic subjects. We showed that 94% of almond-allergic patients had IgE to at least one of
254 those three allergens and that most patients displayed IgE reactivity to Pru du 6 (83%).

255 The main finding of our study was that sensitization to almond legumin Pru du 6 was highly specific
256 for almond allergy. Notably, sensitization to other allergenic legumins from peanut, walnut, hazelnut,
257 and cashew, was similar in almond allergic-and almond-tolerant groups, which may qualify Pru du 6
258 as a specific biomarker for symptomatic almond allergy. The degree of sequence identity between Pru
259 du 6 and the other legumins is low, ranging from 41% to 51% ³², but future inhibition experiments
260 will be necessary to evaluate the extent of cross-reactivity between allergens from almond and other
261 nuts.

262 Furthermore, we evaluated the diagnostic value of molecular diagnosis of almond allergy with regard
263 to specificity compared with that of an extract-based diagnosis. Because there is currently no
264 commercially available diagnostic tool, measurement of sIgE to almond was performed using an in-
265 house developed quantitative ELISA. In line with other studies ⁷⁻¹¹, we found a high sensitization
266 frequency to almond extract not only among almond-allergic, but also almond-tolerant patients and
267 that almond-allergic patients had significantly higher median sIgE levels to almond extract. Our data
268 demonstrate that positive sIgE to almond extract was sensitive (94%), but not specific (33%). At the
269 same threshold level, Pru du 6 maintained high sensitivity (83%) but provided superior specificity
270 (78%). These results clearly demonstrate that the use of single almond components increases the
271 diagnostic specificity compared to extract.

272 This observed improvement is in accordance with other component-resolved diagnosis studies
273 showing that measurement of specific IgE to allergens from hazelnut (Cor a 14), walnut (Jug r 1) or
274 cashew (Ana o 3) improved specificity compared with whole extracts ^{13,15,33}.

275 According to the above-mentioned studies and current state of knowledge, 2S albumins seem to be
276 most important and potent allergens not only in peanut ¹², but also in hazelnut, walnut, and cashew.
277 Compared to almond allergy, those allergies are common and often severe. One could speculate that
278 the distinct allergen composition of almond, in particular the absence of a 2S albumin, might give rise
279 to usually uncommon and mild reactions to almond.

280 The limitations of this study are its monocentric retrospective design and the rather small size of the
281 sample consisting mainly of children. Our findings need to be replicated in other populations,
282 different geographic areas and larger cohorts. For example, in birch endemic areas, almond allergy is
283 often associated with birch pollinosis which may be caused by a cross-reacting Bet v 1-homologue
284 ^{34,35}. Although evidence for a Bet v 1-homologue in almond was provided at the mRNA level, to our
285 knowledge no one has yet confirmed presence of a corresponding protein in almond kernels. In our
286 study, we also could not detect it by immunoblot at the protein level using sera from almond allergic
287 patients containing Bet v 1-specific IgE. Identification of a Bet v 1-homologue in almond requires
288 further studies.

289 Further, interpretation of our findings is limited by the fact that the two groups, almond allergic and
290 almond tolerant subjects, were not comparable by age. The almond allergic group consisted mainly of
291 children, while the almond tolerant group included mostly adolescents and adults. Although the
292 results of a previous study using sera of adult almond allergic patients identified Pru du 6 as a major
293 allergen¹⁹, validation in adults should be the focus of future studies on almond allergy. However,
294 despite these limitations, as being an exploratory in-depth analysis of almond allergens and
295 sensitization profiles of almond-allergic subjects, our results will be of much help for subsequent
296 studies specifically aimed at establishing predictive protocols in even larger cohorts.

297 In conclusion, we found marked differences in allergen composition of almond compared to other tree
298 nuts. Our study provides the first comprehensive evaluation of the sensitization profiles of almond-

allergic patients using a panel of allergens isolated from almond kernels. We showed that the sensitization profile of almond-allergic individuals is dominated by sensitization to almond-specific allergens, particularly Pru du 6, which may potentially assist in effectively discriminating almond-allergic from tolerant patients.

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Author contributions

SK performed experiments, analyzed data, and prepared the manuscript. MB designed and supervised the study. CH, SC, SBS, DK, LEK, and AJL undertook patient recruitment, data collection and were involved in data analysis. HB, KCN, MB and CH critically revised the manuscript.

Conflict of interest statement

Dr. Chinthrajah reports grants from NIAID, CoFAR, Aimmune, DBV Technologies, Astellas, Regeneron, an Advisory member for Alladapt, Genentech, Novartis, and receives personal fees from Before Brands outside the submitted work. Dr. Nadeau reports grants from National Institute of Allergy and Infectious Diseases (NIAID), grants from National Heart, Lung, and Blood Institute (NHLBI), grants from National Institute of Environmental Health Sciences (NIEHS), grants from Food Allergy Research & Education (FARE), other from World Allergy Organization (WAO), other from Cour Pharma, other from Before Brands, other from Alladapt, other from Latitude, other from IgGenix, other from Immune Tolerance Network (ITN), other from National Institutes of Health (NIH) clinical research centers, outside the submitted work; In addition, Dr. Nadeau has a patent Inhibition of Allergic Reaction to Peanut Allergen using an IL-33 Inhibitor pending, a patent Special Oral Formula for Decreasing Food Allergy Risk and Treatment for Food Allergy pending, a patent Basophil Activation Based Diagnostic Allergy Test pending, a patent Granulocyte-based methods for detecting and monitoring immune system disorders pending, a patent Methods and Assays for Detecting and Quantifying Pure Subpopulations of White Blood Cells in Immune System Disorders

326 pending, a patent Mixed Allergen Compositions and Methods for Using the Same pending, and a
327 patent Microfluidic Device and Diagnostic Methods for Allergy Testing Based on Detection of
328 Basophil Activation pending. The rest of the authors declare that they have no relevant conflict of
329 interest related to this study.

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Table 1A Almond allergic (1-18) patients: clinical characteristics and IgE sensitization pattern as determined by quantitative ELISA

Pat No.	Almond allergy confirmed by	Sex/ Age	Symptoms to almond	Other food allergies	sIgE (kU/L)						
					Extract	Pru du 6	Pru du 8	Pru du 10	Bet v 1	Phl p 12+	Pru p 3+
1	DBPCFC	M/7	pruritus, rash, angioedema, nasal congestion	peanut, cashew, egg, milk	7.1	6.7	≤ 0.35	≤ 0.35	0.6	0	≤ 0.35
2	DBPCFC	F/12	urticaria	hazelnut	183.8	148.1	13.4	75.7	25.5	0	≤ 0.35
3	DBPCFC	M/8	rash	peanut, cashew, hazelnut, walnut	125.9	69.4	159.1	33.1	2.1	0.7	3.2
4	DBPCFC	F/4	rash	peanut, milk, wheat	7.5	11.5	≤ 0.35	0.9	≤ 0.35	≤ 0.35	≤ 0.35
5	DBPCFC	F/5	angioedema	peanut, egg, walnut	6.3	5.9	≤ 0.35	≤ 0.35	1.0	≤ 0.35	≤ 0.35
6	DBPCFC	F/10	urticaria	cashew, hazelnut, walnut	0.4	≤ 0.35	≤ 0.35	≤ 0.35	21.8	≤ 0.35	≤ 0.35
7	DBPCFC	M/6	urticaria, rash	peanut, cashew, hazelnut, walnut	8.0	6.5	≤ 0.35	1.0	≤ 0.35	1.7	1.1
8	DBPCFC	F/11	urticaria	peanut, cashew, hazelnut, walnut	317.2	181.7	44.5	24.2	≤ 0.35	≤ 0.35	≤ 0.35
9	DBPCFC	F/7	urticaria	egg	2.7	2.0	1.3	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35
10	DBPCFC	F/12	urticaria	cashew, hazelnut, walnut	12.6	3.6	≤ 0.35	14.4	14.2	≤ 0.35	≤ 0.35
11	DBPCFC	F/9	throat tightness, abdominal pain	none	27.4	8.7	1.5	4.0	3.6	≤ 0.35	≤ 0.35
12	DBPCFC	M/12	urticaria, angioedema	none	2.2	1.1	1.3	≤ 0.35	6.0	1.5	≤ 0.35
13	DBPCFC	M/15	urticaria	none	21.1	6.0	54.1	2.0	33.4	≤ 0.35	≤ 0.35
14	DBPCFC	M/9	urticaria	none	73.7	81.1	0.5	95.1	18.7	≤ 0.35	≤ 0.35
15	(positive		angioedema	peanut, hazelnut,	38.2	8.7	≤ 0.35	0.9	≤ 0.35	≤ 0.35	9.6

+IgE		history)			walnut, cashew, pecan,							
	16	(positive history)	M/4	urticaria	peanut, cashew, pistachio	19.3	22.9	≤ 0.35	3.3	7.0	≤ 0.35	≤ 0.35
	17	(positive history)	F/18	angioedema, oropharyngeal pruritus and mild erythema	peanut	1.6	≤ 0.35	≤ 0.35	0.4	≤ 0.35	3.1	3.4
	18	(positive history)	F/48	angioedema, oropharyngea pruritus and mild erythema	peanut, walnut	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	2.6	≤ 0.35	2.1

specific to Bet v 1, Phl p 12, and Pru p 3 were measured as representatives of an almond Bet v 1-homologue, almond profilin and almond nsLTP, respectively

Table 1B Almond-tolerant (19-36) and non-atopic (37-40) patients: clinical characteristics and IgE sensitization pattern as determined by quantitative ELISA

Pat No.	Almond allergy*	Sex/ Age	Symptoms to almond	Other food allergies	sIgE (kU/L)						
					Extract	Pru du 6	Pru du 8	Pru du 10	Bet v 1+	Phl p 12+	Pru p 3+
19	no	F/31	none	peanut, hazelnut, walnut, poppy seed	4.2	≤ 0.35	≤ 0.35	≤ 0.35	17.1	≤ 0.35	≤ 0.35
20	no	F/22	none	peanut, hazelnut, lupine, peach, kiwi	1.4	0.9	≤ 0.35	3.3	≤ 0.35	≤ 0.35	≤ 0.35
21	no	M/7	none	peanut, hazelnut, walnut, milk	3.2	11.3	0.41	1.8	21.3	≤ 0.35	≤ 0.35
22	no	M/18	none	peanut	≤ 0.35	1.0	≤ 0.35	≤ 0.35	4.3	≤ 0.35	≤ 0.35
23	no	M/17	none	hazelnut, walnut, milk, egg	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	25.4	≤ 0.35	≤ 0.35
24	no	M/20	none	hazelnut, macadamia	0.4	≤ 0.35	≤ 0.35	0.6	≤ 0.35	≤ 0.35	≤ 0.35
25	no	F/27	none	peanut, walnut	2.0	1.6	≤ 0.35	0.9	6.0	≤ 0.35	≤ 0.35
26	no	F/9	none	peanut, hazelnut, walnut, cashew	4.1	≤ 0.35	≤ 0.35	1.4	≤ 0.35	≤ 0.35	≤ 0.35
27	no	F/37	none	hazelnut walnut,	0.8	≤ 0.35	≤ 0.35	0.7	4.4	≤ 0.35	16.3
28	no	M/28	none	peanut	0.6	≤ 0.35	≤ 0.35	≤ 0.35	3.1	≤ 0.35	≤ 0.35
29	no	F/31	none	peanut, hazelnut, walnut	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	7.9	≤ 0.35	≤ 0.35
30	no	M/10	none	peanut, apple	≤ 0.35	≤ 0.35	≤ 0.35	0.5	6.7	≤ 0.35	≤ 0.35
31	no	F/26	none	peanut, hazelnut, walnut	0.8	≤ 0.35	≤ 0.35	≤ 0.35	2.0	≤ 0.35	≤ 0.35
32	no	F/21	none	peanut, hazelnut, walnut	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	15.3	≤ 0.35	≤ 0.35
33	no	F/42	none	walnut	0.45	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	3.4
34	no	M/18	none	peanut	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	0.9	≤ 0.35	≤ 0.35
35	no	F/48	none	hazelnut, walnut	0.4	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35
36	no	F/30	none	peanut, hazelnut	0.5	≤ 0.35	≤ 0.35	≤ 0.35	0.5	≤ 0.35	≤ 0.35
37	no	F/48	none	none	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	0.6	≤ 0.35	≤ 0.35
38	no	M/29	none	none	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	25.5	≤ 0.35	≤ 0.35
39	no	M/32	none	none	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	2.1	≤ 0.35	≤ 0.35

40	no	M/50	none	none	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35	≤ 0.35
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*based on a detailed questionnaire all subjects consumed almonds regularly without allergic symptoms

[†]IgE specific to Bet v 1, Phl p 12, and Pru p 3 were measured as representatives of an almond Bet v 1-homologue, almond profilin and almond nsLTP, respectively

Table 2 Sensitivity and specificity of different cut-off sIgE levels for almond extract, Pru du 6, Pru du 8, and Pru du 10

Test cut off (kUA/L)	Sensitivity (%)	Specificity (%)	Youden index
Almond sIgE			
≥0.1	100	11	0.11
≥0.35*	94	33	0.28
≥5.2†	72	100	0.72
Pru du 6 sIgE			
≥0.1	89	56	0.50
≥0.35*	83	78	0.61
≥1.8†	77	94	0.72
Pru du 8 sIgE			
≥0.1	45	83	0.29
≥0.35*†	44	100	0.44
Pru du 10 sIgE			
≥0.1	72	50	0.22
≥0.35*	67	61	0.28
≥0.75†	61	78	0.38

*clinically used

†calculated

points

cutoff value

optimal cutoff

Figure Legends

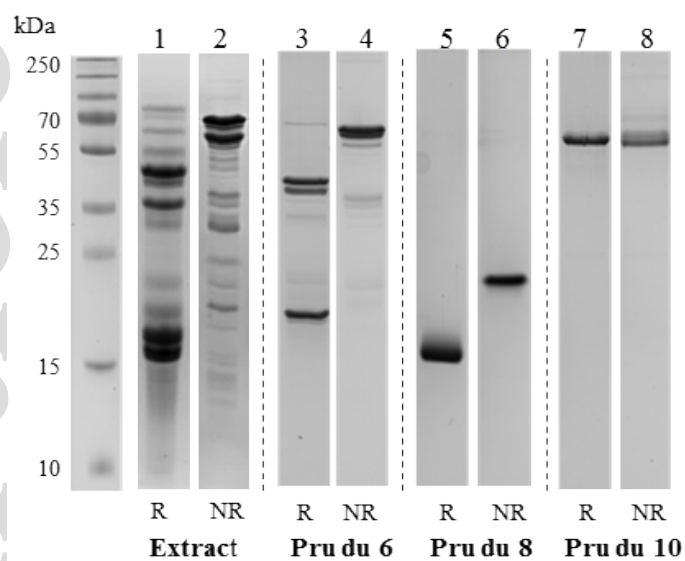
Figure 1. Coomassie-stained 15% SDS-PAGE of almond protein extract and purified allergens separated under reducing (R) and non-reducing (NR) conditions.

Figure 2. IgE immunoblots of almond extract and allergens under reducing (R) and non-reducing (NR) conditions. IgE reactivity of pooled sera (SP) from 4 almond-allergic patients (sera: 4, 5, 10, and 16). To inhibit CCDs specific antibodies serum pool was preincubated with HRP (+HRP). IgE inhibition of the serum pool (SP inhib.) was performed using a mix of Pru du 6, Pru du 8 and Pru du 10. As a negative control, a pool of 2 sera (NHS pool) from atopic subjects (sera: 43 and 44) was used.

Figure 3. Specific IgE concentrations (kU_A/L) to almond extract and individual purified allergens in sera from almond allergic, almond tolerant and other atopic subjects. Horizontal bars indicate medians $\pm$ interquartile ranges. IgE values ≤ 0.01 kU_A/L were set to 0.01 kU_A/L. Dotted lines indicate the 0.10 kU_A/L and 0.35 kU_A/L cut off levels. Differences between the patient groups were analyzed by nonparametric Mann-Whitney U test. *P $\leq$.05, ***P $\leq$.001, **** P $\leq$.0001.

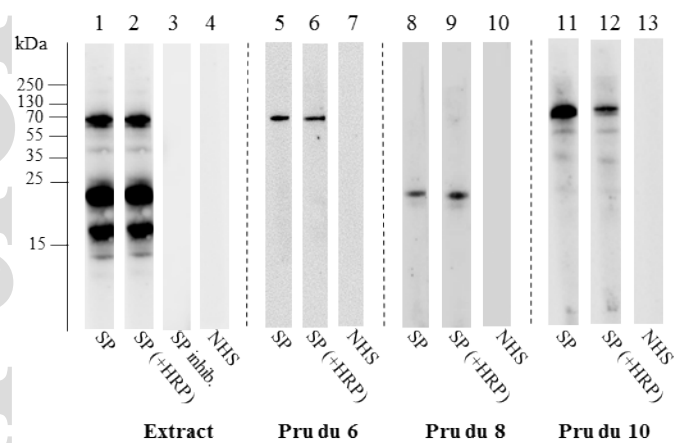
Figure 4. Percentages of sensitization of almond allergic and almond tolerant subjects to Pru du 6 homologous from , walnut (Jug r 4), peanut (Ara h 3), cashew (Ana o 2), and hazelnut (Cor a 9).

Figure 5. Receiver operating characteristic curves for sIgE to almond extract, Pru du 6, Pru du 8 and Pru du 10 with regard to discrimination between almond allergy and tolerance of almond.



revised_Figure 1_Kabasser et al.

A) IgE immunoblots (non-reducing)



B) IgE immunoblots (reducing)

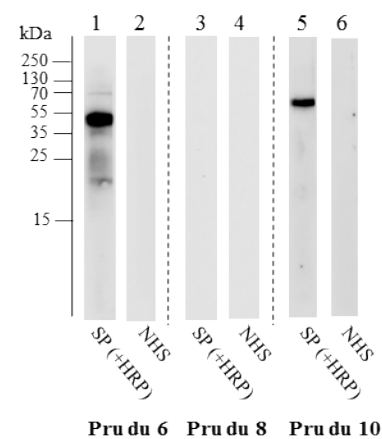


Figure 2_Kabasser et al.

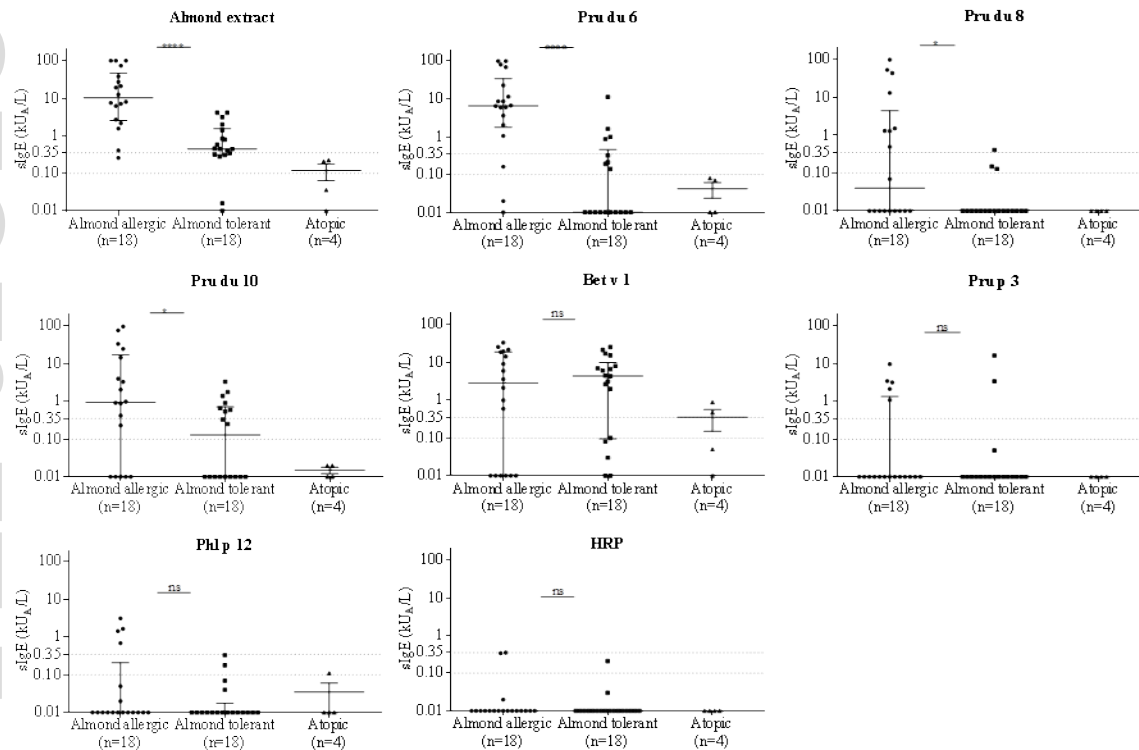


Figure 3_Kabasser et al.

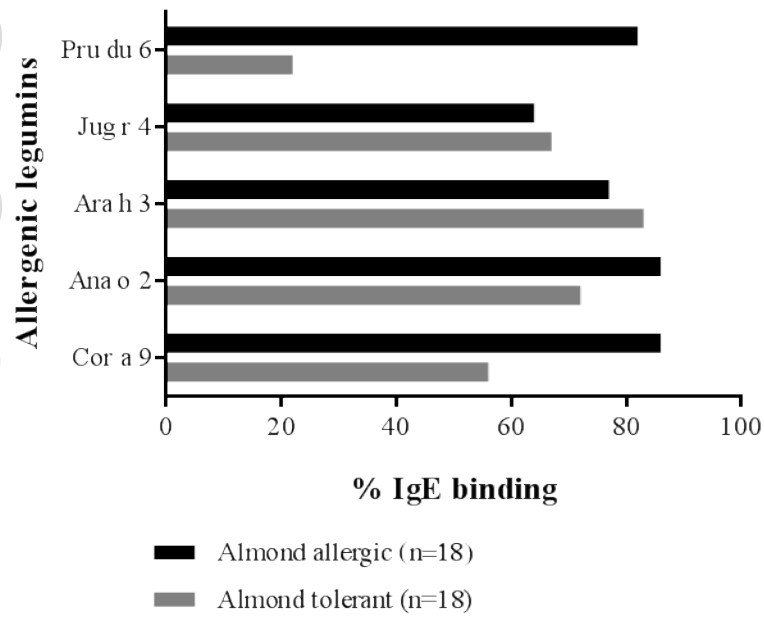


Figure 4_Kabasser et al.

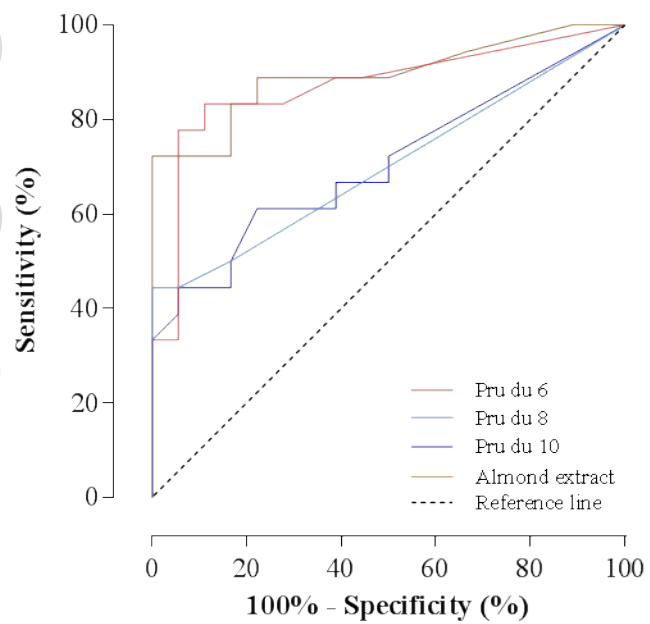


Figure 5_Kabasser et al.

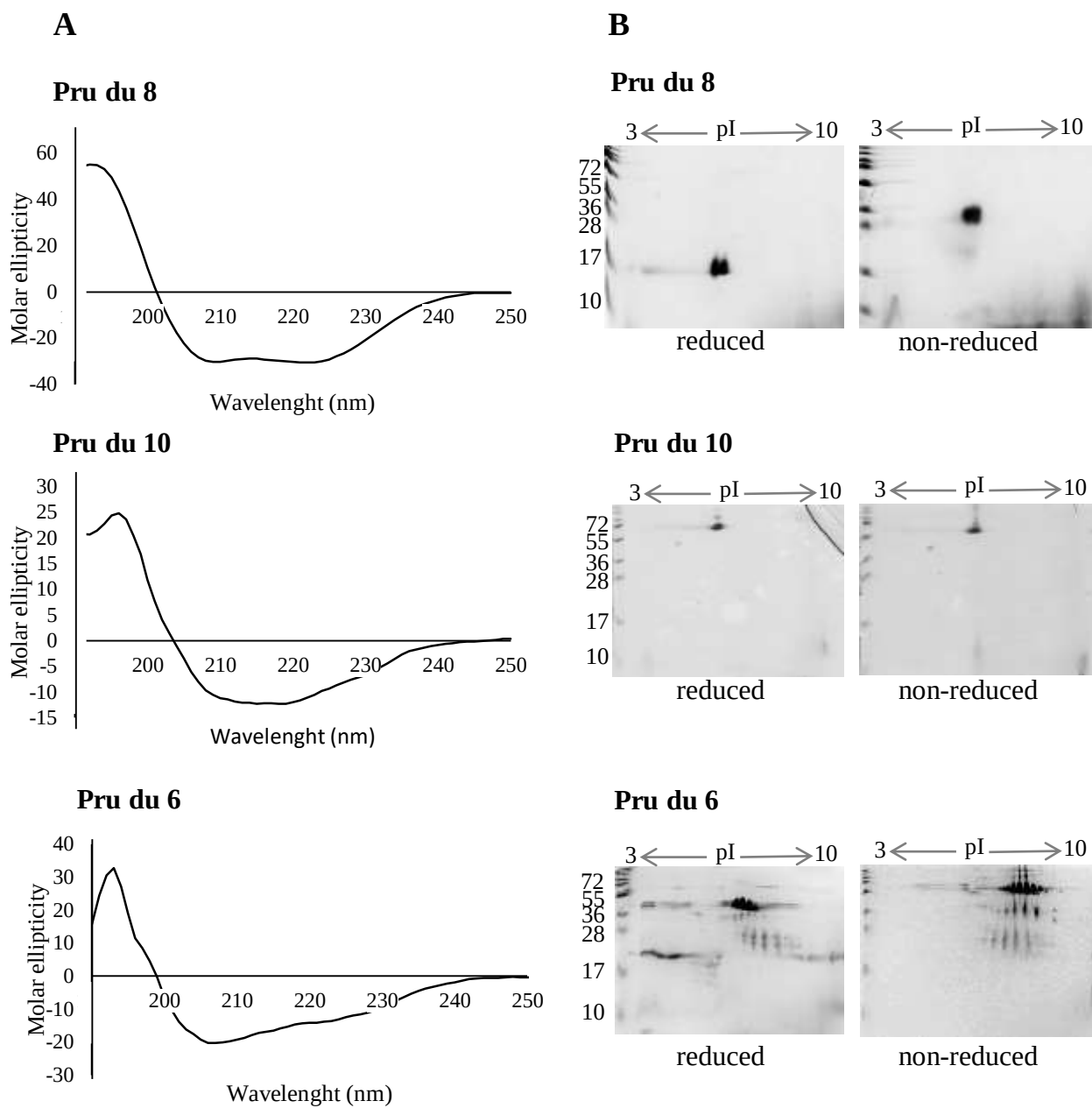


Figure S1_Kabasser et al.

Supplementary Methods and Figures

Identification of Pru du 6 as a marker allergen for almond allergy

Short title: Molecular almond allergy diagnosis

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24 **Materials and Methods**

25 **Materials**

26 Almond used for the preparation of protein extracts were all purchased from local supermarkets.
27 HiPrep Sephacryl S-200 HR (26/60), HiTrap Con A 4B (5 ml), Mono Q 5/50 GL (1 ml), HiTrap
28 DEAE FF (5 ml), and Q Sepharose Fast Flow packing material were from GE Healthcare
29 (Uppsala, Sweden) and Reverse phase-HPLC Jupiter C18 semi-preparative column was from
30 Phenomenex (Torrance, CA, USA). Standard chemicals were purchased from Merck (Darmstadt,
31 Germany) unless stated otherwise.

32 **Preparation of almond extract used for quantitation of almond-specific IgE**

33 Almond kernels were ground, defatted with n-hexane and proteins were extracted from defatted
34 flour with PBS, containing 3% polyvinyl-polypyrrolidone and protease inhibitor cocktail tablets
35 (Roche Molecular Biochemicals, Mannheim, Germany).

36 **Purification of almond allergens**

37 *Purification of Pru du 6*

38 Almond protein extract, prepared as described above, was loaded onto a HiPrep Sephacryl S-200
39 HR gel filtration column equilibrated with 20 mM Tris/HCl, pH 7.6, containing 1 M NaCl.
40 Proteins were eluted at a flow rate of 1.3 ml/min and fractions of 5 ml were collected. Fractions
41 containing high molecular weight proteins were pooled and glycosylated proteins were separated
42 using a HiTrap Con A 4B chromatography column equilibrated in 20 mM Tris/HCl, pH 7.5,
43 containing 0.5 M NaCl. Subsequently, the flow-through containing Pru du 6 was dialyzed
44 overnight at 4°C against 20 mM Tris/HCl, pH 8.0, and further separation was performed using

anion exchange chromatography on a Q-Sepharose column. Bound proteins were eluted using a linear gradient of increasing NaCl concentration (from 0.1 to 0.5 M) over 10 column volumes.

Purification of Pru du 10

Purification of the mandelonitrile lyase was performed according to the protocol for the isolation of 7S globulins from Brazil nut as described by Sharma et al ¹. Briefly, protein extract was loaded onto a HiPrep Sephacryl S-200 HR column equilibrated with PBS, containing 1 M NaCl. The proteins were eluted with a flow rate of 1.3 ml/min over 300 minutes and fractions of 5-10 ml were collected. The fractions containing mandelonitrile lyase were pooled and dialyzed against ddH₂O for 48 h at 4 °C. After lyophilization of the sample, the proteins were dissolved in 20 mM Tris/HCl, pH 8.1, containing 0.1 M NaCl and applied to a HiTrap DEAE FF (5 ml) column. Proteins were eluted by a linear gradient of NaCl (0-0.4 M). Fractions corresponding to the main peak were pooled and further subjected to ConA 4B affinity chromatography, as described for the purification of Pru du 6. Mandelonitrile lyase was eluted in one fraction by the addition of 0.5 M alpha-methyl mannopyranoside (Sigma Aldrich, St. Louis, MS, USA).

Purification of Pru du 8

Proteins were extracted from defatted almond flour by stirring for 1 h at RT in ddH₂O (1:7) (w/v). The slurry was centrifuged (38.900 x g, at RT for 30 min) and the obtained supernatant was cooled to 0 °C prior to addition of cold methanol to 60% (v/v) to precipitate globulins. After stirring at 4 °C for 30 min, precipitated proteins were pelleted by centrifugation (4320 x g, at 4 °C for 20 min). Pru du 8, present in the supernatant, was precipitated overnight at -20 °C by 75% acetone (v/v) followed by centrifugation (7690 x g, at 4 °C for 45 min). The precipitated proteins were dissolved in 20 mM Tris/HCl, pH 8.0, and loaded onto a Mono Q column (1 ml), equilibrated in the same buffer. Bound proteins were eluted with a flow rate of 1 ml/min by

increasing the concentration of NaCl from 0 to 0.3 M in the mobile phase over a time of 20 min. Fractions containing Pru du 8 were pooled and applied onto a RP-HPLC Jupiter C18 semi-preparative column (250 x 10 mm). Solvent A was 0.1% (v/v) trifluoro acidic acid (TFA) and solvent B was water/acetonitrile (10:90 v/v) with 0.085% (v/v) TFA. The column was equilibrated with 10% solvent B and the elution performed with the following gradient: 5 min 10% solvent B, 7 minutes 23% B, followed by a linear gradient of 23-80% B within 45 minutes.

Other allergens used in the study

Previously developed purification protocols were used for the preparation of recombinant Bet v 1 1.0101² and natural allergens Pru p 3, Ara h 3, Ana o 2, Cor a 9 and Jug r 4³⁻⁶.

Circular dichroism (CD) spectroscopy

CD spectra of Pru du 6, Pru du 8, and Pru du 10 (0.2 mg/ml) in 10 mM sodium phosphate buffer, pH 7.5, were recorded in the range of 190-250 nm at room temperature on a J-810S spectropolarimeter (Jasco International, Tokyo, Japan) using a 1 mm path length quartz cell. The average of 5 spectra, recorded at 100 nm/min with a 2 s time constant, 0.5 nm resolution, and sensitivity of ± 100 mdeg, was calculated.

Protein identification by Nano-LC ESI Orbitrap MS/MS and N-terminal sequencing

Five mikrograms protein of each sample was reduced with 50 mM DTT and alkylated with 200 mM iodoacetamide. Proteins were digested over night at 37 °C using Trypsin/LysC Mix (Promega, Madison, WI) in a ratio of 1:25 (Protease:Protein). Before LC-MS analysis peptide extracts were desalted and cleaned up using C18 spin tips according to the manufacturers' protocol.

Bands from Coomassie blue stained 1D gel corresponding to IgE reactive bands on 1D blot were manually excised. After washing and destaining the proteins in the band were reduced with dithiothreitol and alkylated with iodoacetamide⁷. Proteins were digested (8h, 37°C) with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI) with a final trypsin concentration of 20 ng/μl in 50 mM aqueous ammonium bicarbonate and 5 mM CaCl₂. Afterwards, peptides were extracted with three changes of 30 μL of 5% trifluoro acidic acid (TFA) in 50% aqueous acetonitrile supported by ultrasonication for 10 min per change. Extracted peptides were dried down in a vacuum concentrator (Eppendorf, Hamburg, Germany) and redissolved in 0.1% aqueous TFA.

Peptides (200 ng) were separated on a nano-HPLC Ultimate 3000 RSLC system (Dionex). Sample pre-concentration and desalting was accomplished with a 5 mm Acclaim PepMap μ-Precolumn (300 μm inner diameter, 5 μm particle size, and 100 Å pore size) (Dionex). For sample loading and desalting 2% acetonitrile in ultra-pure H₂O with 0.05% TFA was used as a mobile phase with a flow rate of 5 ml/min. Separation of peptides was performed on a 25 cm Acclaim PepMap C18 column with a flow rate of 300 nl/min. For mass spectrometric analysis, the LC was directly coupled to a high-resolution Q Exactive HF Orbitrap mass spectrometer. MS1 spectra were collected in the range 350-2000 m/z for 50 ms. The top 10 intense ions were subjected to Orbitrap for further fragmentation. MS2 spectra were collected in the range 200-2000 m/z for 50 ms. Ions with charge state +1, +7, +8 and >+8 were excluded. Precursor ions were dynamically excluded from reselection for 30 s. Spectra were searched in UniProt/Trembl (downloaded from the publicly available servers (<http://www.uniprot.org>)).

N-terminal sequencing

N-terminal sequencing of purified Pru du 8 by Edman degradation was carried out by the Proteome Factory (Berlin, Germany).

IgE Immunoblotting

Reduced or non-reduced protein samples (2 µg of pure protein or 20 µg of extracts) were electrophoresed on 15% SDS-PAGE and blotted onto 0.2 µm nitrocellulose membranes. Afterwards, membranes were cut into strips and blocked with Tris-buffered saline (TBS), pH 7.4, containing 3% BSA and 0.05% Tween 20. Subsequently, the strips were incubated overnight at 4°C with pooled sera of almond allergic or non-atopic controls (diluted 1:10-1:20). Before adding to the strips, pooled sera of almond allergic patients were incubated for 2 h at room temperature with buffer or horseradish peroxidase (HRP) (Sigma-Aldrich, St Louis, Mo, USA) at a final concentration of 100 µg/ml to inhibit the binding of antibodies specific for cross-reactive carbohydrate determinants (CCDs). For inhibition of IgE binding to the blotted almond extract, sera were pre-incubated with a mixture of Pru du 6, Pru du 8 and Pru du 10 (100 µg/ml of each protein). For detection of bound IgE by enhanced chemiluminescence (ECL), membrane strips were incubated with a 1:3000 diluted HRP conjugated anti-human IgE antibody (KPL, Gaithersburg, MD, USA) followed by incubation with Clarity maxTM Western ECL Substrate (Biorad, Hercules, CA, USA) according to the manufacturers' protocol. 2D electrophoresis was performed as previously described⁹.

IgE ELISA

Wells of microtiter plates (Maxisorp; Nalge Nunc International, Roskilde, Denmark) were coated with 0.3 µg of purified protein or 2 µg of almond extract. After blocking, individual patients'

sera were diluted in TBST with 0.5% BSA and applied in duplicates overnight. Before adding to the plates, all sera were pre-incubated with horseradish peroxidase (100 µg/ml) (Sigma-Aldrich, St Louis, Mo, USA) to inhibit the binding of antibodies specific for cross-reactive carbohydrate determinants (CCDs). Bound IgE was detected with an alkaline phosphatase-conjugated mouse anti-human IgE antibody (BD Pharmingen, San Jose, Ca, USA) followed by incubation with Sigma FAST p-nitrophenyl phosphate tablets (Sigma-Aldrich, St Louis, Mo, USA). Absorbance was measured at 405 nm.

Quantitation of IgE specific to almond extract and allergens was performed using Bv16 IgE, a Bet v 1-specific monoclonal IgE antibody (Absolute Antibody Ltd, Redcar, UK), in concentrations between 0.17 and 10 ng/ml, and Bet v 1 (30 ng/well) as catching protein. The limit of detection was defined as the standard concentrations yielding OD-values exceeding the buffer controls.

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174

Table S1 Comparison of total and specific IgE in subjects allergic and tolerant to almond

IgE (kU/L)		Almond allergic (n=18)	Almond tolerant (n=18)	P value	Atopic controls (n=4)
Total IgE	Mean $\pm$ SD	930.6 $\pm$ 741.3	755.9 $\pm$ 666.6	0.52	58.5 $\pm$ 96.4
	Median; range	700.5 (75-2710)	677.5 (56.0-1816.0)		12.5 (6.0-203.0)
Almond	No.	17 (94%)	12 (67%)	< 0.0001	0
	Mean $\pm$ SD	50.3 $\pm$ 85.2	1.6 $\pm$ 1.5		n/a
	Median; range	12.6 (0.4-317.2)	0.8 (0.4-4.2)		n/a
Pru du 6	No.	15 (83%)	4 (22%)	< 0.0001	0
	Mean $\pm$ SD	37.6 $\pm$ 57.4	3.7 $\pm$ 5.1		n/a
	Median; range	8.7 (1.1-181.7)	1.3 (0.9-11.3)		n/a
Pru du 8	No.	8 (44%)	1 (6%)	0.01	0
	Mean $\pm$ SD	34.5 $\pm$ 54.6	n/a		n/a
	Median; range	7.4 (0.5-159.0)	n/a		n/a
Pru du 10	No.	12 (67%)	7 (39%)	0.03	0
	Mean $\pm$ SD	21.2 $\pm$ 32.0	1.3 $\pm$ 1.0		n/a
	Median; range	3.6 (0.4-95.1)	0.9 (0.5-3.3)		n/a
Bet v 1	No.	12 (67%)	15 (83%)	0.70	2
	Mean $\pm$ SD	13.0 $\pm$ 10.9	9.0 $\pm$ 7.5		0.7 $\pm$ 0.3
	Median; range	11.6 (0.6-33.4)	6.7 (2.0-25.4)		n/a
Phl p 12	No.	5 (28%)	1 (6%)	0.57	0
	Mean $\pm$ SD	1.5 $\pm$ 1.0	n/a		n/a
	Median; range	1.5 (0.7-3.1)	n/a		n/a
Pru p 3	No.	5 (28%)	2 (11%)	0.52	0
	Mean $\pm$ SD	1.1 $\pm$ 2.4	1.1 $\pm$ 3.9		n/a
	Median; range	3.2 (1.1-9.6)	9.8 (3.4-16.3)		n/a
Peanut	No.	14 (78%)	14 (78%)	0.58	0
	Mean $\pm$ SD	21.5 $\pm$ 19.2	14.6 $\pm$ 13.0		n/a
	Median; range	14.2 (2.11-61.4)	11.3 (0.7-48.2)		n/a
Hazelnut	No.	11 (61%)	9 (50%)	0.48	0
	Mean $\pm$ SD	5.1 $\pm$ 5.0	3.6 $\pm$ 2.7		n/a
	Median; range	4.0 (0.7-18.0)	2.8 (0.5-8.9)		n/a
Walnut	No.	11.0 (61%)	6 (33%)	0.32	0
	Mean $\pm$ SD	20.3 $\pm$ 18.8	11.9 $\pm$ 11.4		n/a
	Median; range	17.5 (0.4-59.9)	9.3 (1.0-25.9)		n/a
Cashew	No.	12 (67%)	4 (22%)	0.003	0
	Mean $\pm$ SD	22.2 $\pm$ 18.1	7.8 $\pm$ 2.3		n/a
	Median; range	17.3 (3.0-55.1)	7.7 (5.2-10.7)		n/a
Brazil nut	No.	7 (39%)	3 (17%)	0.03	0
	Mean $\pm$ SD	2.5 $\pm$ 2.3	6.8 $\pm$ 10.9		n/a
	Median; range	2.3 (0.4-6.6)	0.6 (0.4-19.4)		n/a

IgE (kU/L)		Almond allergic (n=18)	Almond tolerant (n=18)	P value	Atopic controls (n=4)
Macadamia	No.	3 (17%)	2 (11%)	0.65	0
	Mean $\pm$ SD	5.3 $\pm$ 3.3	4.8 $\pm$ 4.6		n/a
	Median; range	5.9 (1.7-8.3)	4.8 (1.6-8.0)		n/a
Ara h 1	No.	13 (72%)	12 (67%)	0.74	0
	Mean $\pm$ SD	19.9 $\pm$ 20.4	17.4 $\pm$ 17.3		n/a
	Median; range	14.8 (1.5-68.0)	12.5 (1.3-52.2)		n/a
Ara h 2	No.	13 (72%)	12 (67%)	0.55	0
	Mean $\pm$ SD	24.6 $\pm$ 20.5	20.1 $\pm$ 16.8		n/a
	Median; range	19.4 (2.4-73.7)	15.8 (1.9-60.1)		n/a
Ara h 3	No.	10 (56%)	10 (56%)	0.86	0
	Mean $\pm$ SD	12.5 $\pm$ 11.5	10.8 $\pm$ 13.7		n/a
	Median; range	11.1 (1.1-36.2)	4.1 (0.4-40.2)		n/a
Cor a 8	No.	1 (6%)	1 (6%)	0.20	0
	Mean $\pm$ SD	n/a	n/a		n/a
	Median; range	n/a	n/a		n/a
Cor a 9	No.	6 (33%)	4 (22%)	0.26	0
	Mean $\pm$ SD	3.7 $\pm$ 4.5	3.2 $\pm$ 3.1		n/a
	Median; range	1.2 (0.5-11.0)	2.1 (0.9-7.6)		n/a
Cor a 11	No.	10 (56%)	3 (17%)	0.17	0
	Mean $\pm$ SD	4.5 $\pm$ 6.3	1.5 $\pm$ 1.0		n/a
	Median; range	1.5 (0.5-20.6)	1.4 (0.7-2.6)		n/a
Cor a 14	No.	5 (28%)	4 (22%)	> 0.9999	0
	Mean $\pm$ SD	5.5 $\pm$ 6.8	8.8 $\pm$ 2.3		n/a
	Median; range	1.4 (0.4-16.3)	8.9 (6.1-11.3)		n/a
Jug r 1	No.	8 (44%)	5 (28%)	0.19	0
	Mean $\pm$ SD	8.1 $\pm$ 7.9	10 $\pm$ 8.5		n/a
	Median; range	6.5 (0.7-22.7)	10.8 (0.5-20.5)		n/a
Jug r 2	No.	10 (56%)	6 (33%)	0.08	0
	Mean $\pm$ SD	13.8 $\pm$ 13.7	5.1 $\pm$ 6.3		n/a
	Median; range	11.8 (0.6-40.2)	2 (0.5-15.7)		n/a
Ana o 3	No.	11 (61%)	4 (22%)	0.01	0
	Mean $\pm$ SD	22.3 $\pm$ 16.9	5.4 $\pm$ 3.7		n/a
	Median; range	20.9 (2.1-56.4)	4.6 (2.2-10.1)		n/a

178 No.: Number of patients with sIgE >0.35 kU_A/L; Mean $\pm$ SD and median, range of sIgE >0.35

179 kU_A/L; n/a: not applicable

Table S2 Sequence coverage (bold) obtained by LC-MS/MS analysis of purified Pru du 6, Pru du 8 and Pru du 10

a) Pru du 6.0101 (Uniprot Accession No.: E3SH28)

10	20	30	40	50
MAKAFVFSLC	LLLVFNGCLA	AR Q S Q LSP Q N	Q C Q L N Q L Q A R	EPDNRI Q A E A
60	70	80	90	100
G Q I ETW N F N Q	E D F Q C A G V A A	S R I T I Q R N G L	H L P S Y S N A P Q	L I Y I V Q G R G V
110	120	130	140	150
L G A V F S G C P E	T F E E S Q Q S S Q	Q G R Q Q E Q E Q E	R Q Q Q Q Q G E Q G	R Q Q G Q Q E Q Q Q
160	170	180	190	200
ERQGRQ Q GRQ	Q Q EEGR Q Q E Q	Q Q G Q Q GR P Q Q	Q Q Q FR Q FDRH	QKTRRIR E G D
210	220	230	240	250
V V A I P A G V A	W S Y N D G D Q E L	V A V N L F H V S S	D H N Q L D Q N P R	K F Y L A G N P E N
260	270	280	290	300
E F N Q Q G Q S Q P	R Q Q G E Q G R P G	Q H Q Q P F G R P R	Q Q E Q Q G S G N N	V F S G F N T Q L L
310	320	330	340	350
A Q A L N V N E E T	A R N L Q G Q N D N	R N Q I I R V R G N	L D F V Q P P R G R	Q E R E H E E R Q Q
360	370	380	390	400
E Q L Q Q E R Q Q Q	G G Q L M A N G L E	E T F C S L R L K E	N I G N P E R A D I	F S P R A G R I S T
410	420	430	440	450
L N S H N L P I L R	F L R L S A E R G F	F Y R N G I Y P H	W N V N A H S V V Y	V I R G N A R V Q V
460	470	480	490	500
V N E N G D A I L D	Q E V Q Q G Q L F I	V P Q N H G V I Q Q	A G N Q G F E Y F A	F K T E E N A F I N
510	520	530	540	550
T L A G R T S F L R	A L P D E V L A N A	Y Q I S R E Q A R Q	L K Y N R Q E T I A	L S S S Q Q R R A V
560				

V

b) vicilin-like antimicrobial peptides 2-2 (Uniprot Accession No.: A0A314YX39)

10	20	30	40	50
MTKAELPLL V	LFLSTLL L A T	SVPSARA Q V T	C E E G C Y S L S D	Q S K V G E C L Q M
60	70	80	H 90	100
C S S H G Q S C E D	R C M R E A R W P Q	Q Q E Q C L R M C R	<u>QQEQGKHLPC</u>	<u>REQCIRSPDR</u>
110	120	130	140	150
EKCERDC Q Q Q	Q G Q G G G R Q C L	Q Q C K M I T R D P	R K R I E C V R R C	M Q V E Q Q G V F Q
160	170	180	190	200
<u>GQQQQQQQV</u>	E Q Q C R Q H C S	E R D P M R Q Q E C	Q E D C V G Q M M Q	Q E Y E Q Q C R S R
M 210	220	230	240	250
<u>CQSERPRREQ</u>	Q E Q C Q E E C T E	K I R Q L E Q C Q E	G C K M Q G Q Y G P	Q Q Q E C Q R M C R
Q 260				
<u>EQFEQGRGIR</u>	M V A			

Underlined sequence represents N-terminus identified by Edman degradation.

Double underlined sequences represent peptides identified by de-novo sequencing.

c) (R)-mandelonitrile lyase 2 (Pru du 10) (Uniprot Accession No.: Q945K2)

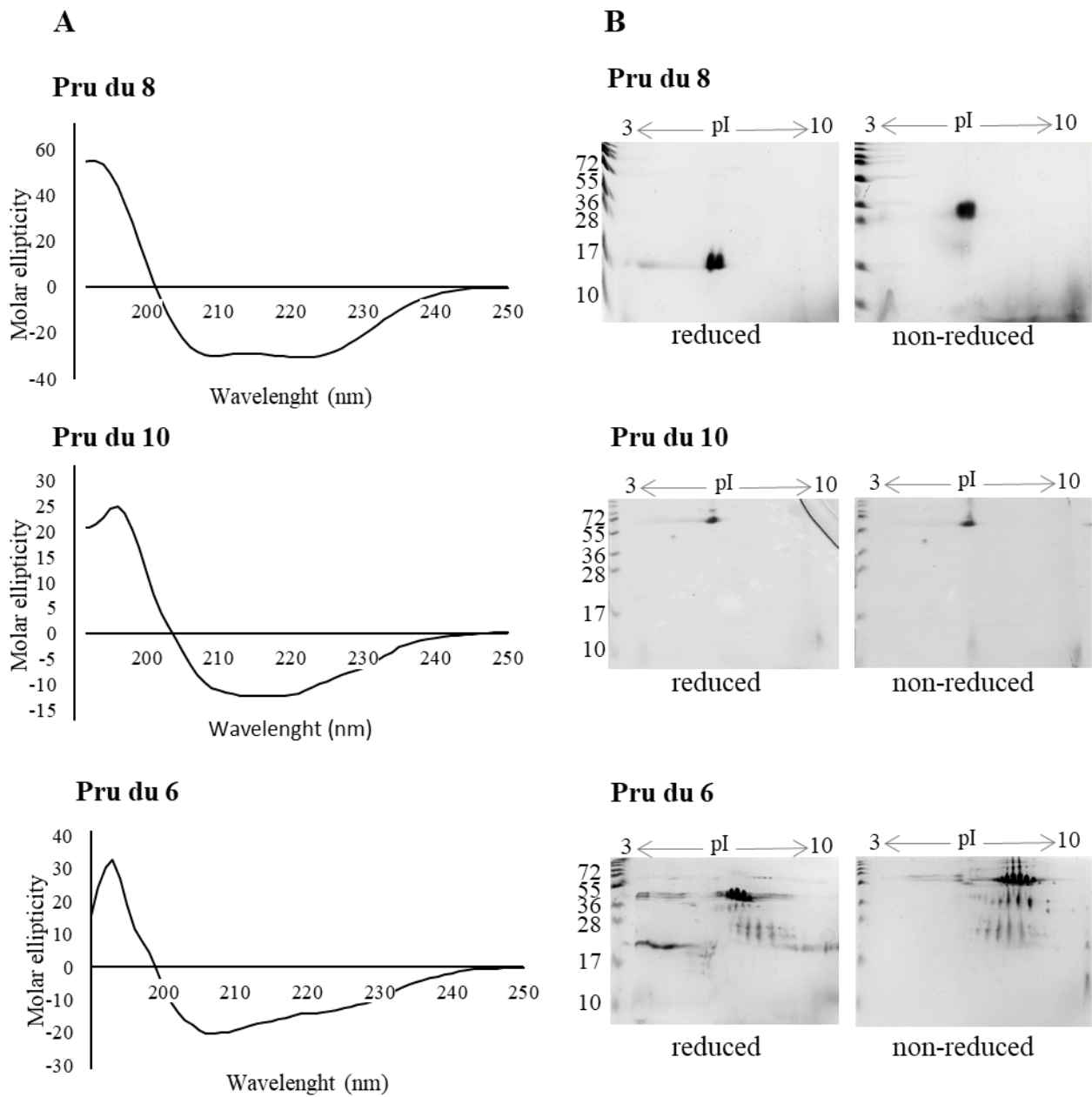
10	20	30	40	50
MEKSTMSAIL	LVLVIFVLHL	QYSEVHSLAT	TSDHDFSYSLS	FAYDATDLEL
60	70	80	90	100
EGSYDYVIVG	GGTSGCPLAA	TLSEKYKVLV	LERGSLPTAY	PNVLTADGFV
110	120	130	140	150
YNLQQEDDGK	TPVERFVSED	GIDNVRGRVL	GGTSIINAGV	YARANTSIYS
160	170	180	190	200
ASGVDWMDL	VNQTYEVED	TIVYKPNSQS	WQSVTK TAF L	EAGVHPNHGF
210	220	230	240	250
SLDHEEGTRI	TGSTFDNKGT	RHAADLLNK	GNSNNLRVGV	HASVEKIIFS
260	270	280	290	300
NAPGLTATGV	IYRDSNGTPH	QAFVRSKGEV	IVSAGTIGTP	QLLLLSGVGP
310	320	330	340	350
ESYLSSLNIP	VVLSPYVVGQ	FLHDNPRNFI	NILPPNPIEP	TIVTVLGISN
360	370	380	390	400
DFYQCSFSSL	PFTTPPFGFF	PSASYPLPNS	TFAHFASK VA	GPLSYGSLTL
410	420	430	440	450
KSSSNVRVSP	NVKFNYYSNL	TDLSHCVSGM	KKIGELSTD	ALKPYKVEDL
460	470	480	490	500
PGVEGFNILG	IPLPKDQTDD	AAFETFCRES	VASYWHYHGG	CLVGKVLGDG
510	520	530	540	550
FRVTGINALR	VVDGSTFPYT	PASHPQGFYL	MLGRYVGIKI	LQERSASDLK
560				
ILDSLKSAAS	LVL			

185 **Figure Legends**

186 **Figure S1.** Analysis of the purified allergens by (A) CD spectroscopy and (B) 2D-
187 electrophoresis.

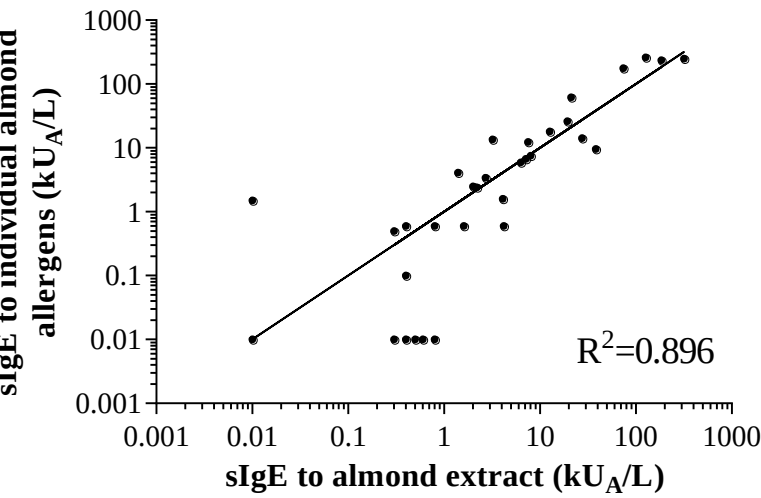
188 **Figure S2.** Correlation between reactivity to almond extract and specific reactivity to the three
189 almond allergens (sum of sIgE values of all individual allergens).

190 **Figure S1**



191
192

Figure S2



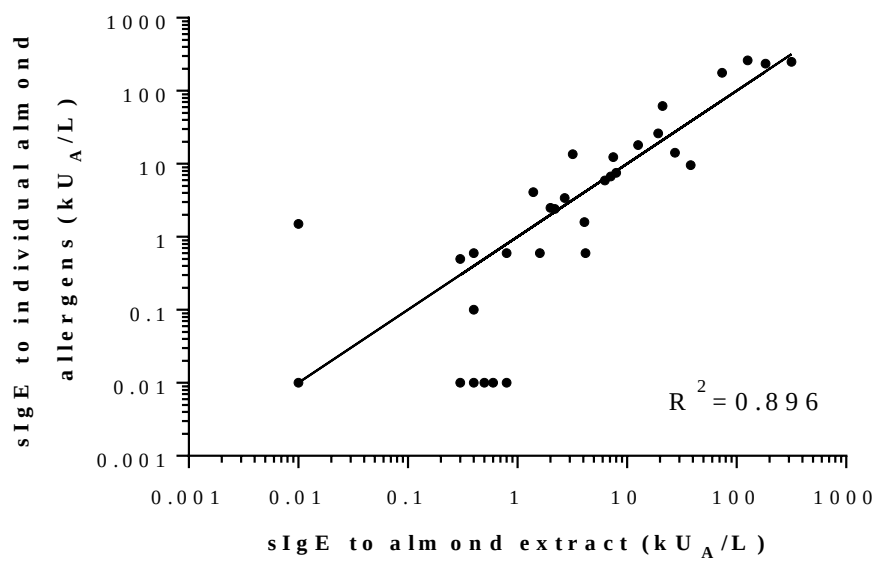


Figure S2_Kabasser et al.