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Identification and characterization of the proteins bound by specific phage-displayed recombinant antibodies (scFv) obtained against Brazil nut and almond extracts

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ABSTRACT

BACKGROUND: Almonds and Brazil nuts are widely consumed allergenic nuts whose presence must be declared according to food labelling regulations. Their detection in food products has been achieved by ELISA methods with recombinant antibodies (scFv) isolated against complete Brazil nut and almond protein extracts. The screening of phage-scFv libraries against complete protein extracts confers a series of advantages over the use of purified proteins, as recombinant proteins might alter their native folding. However, using this strategy, the nature of the target detected by phage-displayed antibodies remains unknown.

RESULTS: Electrophoretic, chromatographic, immunological and spectrometric techniques revealed that the Brazil nut (BE95) and almond (PD1F6 and PD2C9) specific phage-scFvs detected conformational epitopes of the Brazil nut and almond 11S globulins, recognized by WHO/IUIS as Ber e 2 and Pru du 6 major allergens. Circular dichroism data indicated that severe heat treatment would entail loss of epitope structure, disabling scFv for target detection.

CONCLUSIONS: The presence of important Brazil nut and almond allergens (Ber e 2 and Pru du 6) in foodstuffs can be determined by using phage-display antibodies BE95, PD1F6 and PD2C9 as affinity probes in ELISA.

KEYWORDS

11S globulin, amandin, almond, Brazil nut, scFv.

21 INTRODUCTION

22 Food allergy has become a serious public health problem in developed countries, with
23 an estimated prevalence of 3% when considering data from food challenges studies
24 from Europe, USA and Australia/New Zealand.¹ Food allergy involves an abnormal
25 immune response to food proteins, triggering the production of specific type E
26 immunoglobulins (IgE) against the allergens. Currently, the only reliable method for
27 preventing food allergic reactions is the total avoidance of the offending food. In this
28 sense, many countries have approved regulations enforcing the declaration of food
29 allergens in foodstuffs. As an example, European Regulation No 1169/2011 provides a
30 list of mandatory particulars to be indicated on the label, which comprises 14 products
31 causing allergies or intolerances: cereals containing gluten, crustaceans, eggs, fish,
32 peanuts, soybeans, milk, tree nuts, celery, mustard, sesame, sulphur dioxide and
33 sulphites, lupine and molluscs.

34 Due to their organoleptic properties and allegedly health benefits, Brazil nut
35 (*Bertholletia excelsa*) and almond (*Prunus dulcis*) are extensively incorporated in food
36 products, so high population exposure is observed.²⁻⁴

37 Therefore, in order to meet legal requirements, food industries and official regulatory
38 agencies need sensitive and accurate techniques that could provide information of the
39 presence of tree nuts in food products. ELISA, the enzyme-linked immunosorbent
40 assay, takes advantage of antibodies that specifically recognize and bind a particular
41 antigen, which can be either a marker protein or the allergen itself.^{5,6} Traditionally,
42 those specific antibodies have been raised in animals.⁷⁻⁹ However, there is a tendency
43 towards the replacement of antibodies raised in animals with antibodies synthesized *in*
44 *vitro*, products of synthetic libraries.¹⁰⁻¹² Filamentous bacteriophage libraries that

45 display antibody fragments fused to one of the phage coat proteins have been
46 successfully used for this purpose.^{13,14}
47 In two previous works,^{15,16} we reported the isolation of a specific scFv against Brazil
48 nut (named BE95) and two specific scFv against almond (named PD1F6 and PD2C9)
49 from the commercial phage library Tomlinson I, using crude protein extracts from
50 Brazil nut or almond as targets to perform the selection procedure. Those phage-clones
51 were successfully used in indirect phage-ELISA to detect the presence of Brazil nut and
52 almond in commercial food products. However, as library screenings were performed
53 employing a whole nut protein extract, the identity of the targets still remains unknown.
54 Hence, the main objective of this work was to identify and characterise the specific
55 protein or proteins from the tree nut extracts detected by each of the scFv antibodies.

56 MATERIALS AND METHODS

57 *Materials and Chemicals*

58 Phage clones BE95, PD1F6 and PD2C9 employed to detect Brazil nut and almond
59 proteins were isolated from Tomlinson I library (Source BioSciences, Nottingham, UK)
60 after two rounds of *biopanning*, in two independent experiments, as described
61 elsewhere.^{15,16}

62 Brazil nut and almond (Marcona cultivar) kernels were acquired from a local retailer in
63 Madrid (Spain). After being shelled separately to avoid cross-contamination, they were
64 stored at -20 °C until further use.

65 All chemicals used in this work were purchased from Sigma-Aldrich (St. Louis, MO,
66 USA) unless otherwise stated.

67 *Preparation of protein extracts*

68 To prepare protein extracts, shelled kernels maintaining the testa were ground to a fine
69 powder using a mortar and pestle, and stored in screw-capped vials at -20 °C until
70 further use. Protein from 2 g of ground Brazil nut or almond was extracted with 20 mL
71 of extraction buffer (0.035 M phosphate buffer containing 1 M NaCl, pH 7.5), by
72 shaking for 1 h at 25 °C in a vertical rotator (HulaMixer Sample Mixer, Life
73 Technologies, Carlsbad, CA). The slurry obtained was centrifuged at 10 000 g for 30
74 min at 4 °C, and supernatant was filtered through a 0.20 µm syringe filter (Sartorius,
75 Göttingen, Germany). Protein content was measured with the bicinchoninic acid (BCA)
76 assay (Thermo Fisher Scientific Inc.) employing bovine serum albumin (BSA) as the
77 standard protein. Aliquots of the protein extracts were kept at -20 °C until used.

78 To assess that phage clones were indeed recognizing proteins and not any other
79 component of the extract, when mentioned, a digestion with two different proteases was
80 performed. Proteinase K treatment was carried out by adding 400 µg of enzyme to
81 500 µL of a 50 µg mL⁻¹ protein extract , and incubating for 1 h at 37 °C. Trypsin
82 digestion was performed by adding 500 µg of enzyme to 450 µL of a 50 µg mL⁻¹ protein
83 extract, and incubating for 30 min at 37 °C. Both reactions were stopped by adding a
84 protease inhibitor cocktail (Halt Protease Inhibitor Single-Use Cocktail, Thermo
85 Scientific), following manufactures' instructions.

86 *SDS-PAGE and western blotting analysis*

87 SDS-PAGE was performed using precast polyacrylamide gels (4-20 % Mini-Protean
88 TGX Gel, Bio-Rad, Hercules, CA, USA). Electrophoresis was run at constant voltage
89 (150 V) using a Mini-Protean Tetra Cell (Bio-Rad) until tracking dye reached the
90 bottom of the gel. Discontinuous native PAGE electrophoresis was performed according
91 to Ornstein (1964),¹⁷ preparing an 8 % resolving gel, pH 8.8, with a 4 % stacking gel,

92 pH 6.8. Continuous native PAGE electrophoresis was performed according to McLellan
93 (1982),¹⁸ with a 6 % gel, pH 7.4. When required, after electrophoresis, half of the gel
94 was stained with Coomassie Brilliant Blue R-250 and the other half was transferred for
95 western blotting analysis into a methanol-activated polyvinylidene difluoride (PVDF)
96 membrane (Immun-Blot PVDF membranes, Bio-Rad) using a Mini Trans-Blot Cell
97 (Bio-Rad). Running conditions were performed at 400 mA for 1 h, using a transfer
98 buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, and 20 % methanol. After
99 transferring the proteins, the membrane was blocked with 5 % (w/v) dry skimmed milk
100 (Central Lechera Asturiana, Spain) in PBS (0.01 M phosphate buffer, 0.0027 M
101 potassium chloride, 0.137 M sodium chloride, pH 7.4) for 1 h at 37 °C. Then, the
102 membrane was washed 3 times with PBS, and incubated with approximately $5 \cdot 10^7$
103 phage particles in 5 % (w/v) dry skimmed milk in PBS overnight at 4 °C. Next day,
104 membrane was washed 3 times with PBS, and incubated with a 1:5000 dilution of
105 HRP/anti-M13 monoclonal mouse antibody (GE Healthcare, München, Germany) in
106 5 % skimmed milk, for 1 h at room temperature. After washing 3 times with PBS, the
107 membrane was incubated with a chemiluminescent substrate (Clarity western ECL, Bio-
108 Rad) at room temperature for 5 min, to visualize bands. Coomassie stained gels and
109 western blotting membranes were scanned using a ChemiDoc XRS system (Bio-Rad).

110 *Size-exclusion chromatography*

111 Size-exclusion chromatography separation was carried out in a fast protein liquid
112 chromatography (FPLC) system (Pharmacia-LKB, Uppsala, Sweden). Protein extract (1
113 mL) of Brazil nut, prepared as described before, was injected into a HiPrep 16/60
114 Sephacryl S-200 HR column (GE, Healthcarre UK Ltd., Buckinghamshire, UK)
115 previously equilibrated with extraction buffer. The flow rate was maintained at 1 mL

116 min⁻¹. Eluted fractions were collected in 1.5 mL tubes and stored at -20 °C until further
117 use. To assess the ability of the scFv to recognize different peaks, protein fractions
118 coming from size-exclusion chromatography were analysed by an indirect-phage
119 ELISA using BE95 phage clone.

120 *Indirect phage-ELISA*

121 Flat-bottom polystyrene 96-multiwell plates (F96 MaxiSorp Nunc immunoplates, Nunc,
122 Denmark) were coated with 100 µL of a 1:5 (v/v) dilution in PBS of each of the
123 different chromatographic peaks, and plates were incubated overnight at 4° C. Next day,
124 plates were washed 3 times, and blocked with 1% ovalbumin in PBS for 1 h at 37 °C.
125 After 3 washes, 100 µL of 1 % ovalbumin in PBS containing $\sim 3 \cdot 10^8$ phage particles
126 were added to each well. After 1 h of incubation at room temperature, plates were
127 washed 10 times. One hundred microliters of a 1:5000 dilution of HRP/anti-M13
128 monoclonal mouse antibody in 1% ovalbumin was added, and plates were incubated at
129 room temperature for an extra hour. Finally, plates were washed 5 times, and 100 µL of
130 tetramethylbenzidine substrate solution was added to each well. Colour development
131 was performed in the dark for 10 min at room temperature, before stopping the reaction
132 with 1 M sulphuric acid. OD₄₅₀ was measured in an iEMS Reader MF (Labsystems,
133 Helsinki, Finland). All washing steps were performed with PBS.

134 *Anion exchange chromatography of amandin*

135 Almond soluble proteins for anion exchange chromatography were obtained from 1 g of
136 ground almond dispersed in 10 mL of ultrapure H₂O (Milli-Q, Merck Millipore, MA,
137 USA). The mixture was shaken in a vertical rotator for 1 h at room temperature. The
138 sample was centrifuged at 10 000 g for 30 min, and the supernatant was filtered through

139 a 0.20 μm syringe filter. Chromatography was performed in an ÄKTA purifier FPLC
140 system (GE Healthcare, Sweden) following the procedure described in Albillos et al.,
141 2008,¹⁹ with some modifications. Briefly, 1 mL of protein extract was diluted in 10 mL
142 of 10 mM Tris-HCl buffer, pH 7.9, and loaded onto a 1 mL Mono Q HR 5/5 anion
143 exchange column (GE Healthcare) previously equilibrated with Tris-HCl buffer. Sample
144 was eluted with Tris-HCl and a gradient of 0-0.3 M NaCl over 20 minutes. Fractions
145 corresponding to different peaks were collected and analysed by indirect phage-ELISA
146 and SDS-PAGE followed by immunoblot.

147 *Analytical Ultracentrifugation*

148 Chromatographic peaks containing the protein of interest were dialyzed against 10 mM
149 phosphate buffer with 2.7 mM KCl and 40 mM NaCl, pH 7.4, and concentrated using
150 Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore, Darmstadt, Germany.) with
151 a nominal molecular weight limit of 10 kDa.

152 Sedimentation velocity experiments were carried out at 45 000 rpm in an Optima XL-A
153 analytical ultracentrifuge (Beckman Coulter Inc., Fullerton, CA), using an AN50Ti
154 rotor and standard cells with double-sector epon-charcoal centrepieces. Measurements
155 were performed at 20 °C with 400 μL of sample at protein concentrations of
156 314 $\mu\text{g mL}^{-1}$ for amandin and 37 $\mu\text{g mL}^{-1}$ for Ber e 2. Differential sedimentation
157 coefficients were calculated by least-squares boundary modeling of the experimental
158 data and corrected to $s_{20,w}$ values with the program SEDFIT,²⁰ using a partial specific
159 volume of 0.73 mL g^{-1} . Solvent density and viscosity at 20 °C were computed using the
160 SEDNTERP program.²¹ The experiments were performed at Instituto de Química-Física
161 Rocasolano, CSIC, Madrid (Spain).

162 *Peptide identification*

163 Electrophoresis gels were stained with Coomassie Brilliant Blue R-250. Gel bands of
164 interest were cut out with a scalpel and immersed in 5 % acetic acid solution. Peptide
165 mass fingerprinting was performed using a 4800 Plus MALDI TOF/TOF Analyser mass
166 spectrometer (AB SCIEX, MA, USA), at the Unidad de Proteómica, Universidad
167 Complutense de Madrid (Spain). Mascot search engine software
168 (<http://www.matrixscience.com>) was employed to interpret mass spectra data into
169 protein identities using the SwissProt database. Search parameters employed were:
170 trypsin enzymatic cleavage, one possible missed cleavage allowed; peptide mass
171 tolerance of ± 80 ppm; fragment mass tolerance of ± 0.3 Da; peptides were assumed to
172 be monoisotopic; carbamidomethyl fixed modification; and methionine oxidation
173 variable modification.

174 *Circular dichroism*

175 Circular dichroism (CD) spectra were recorded with a Jasco-810 spectropolarimeter
176 (JASCO Ltd, Spain) equipped with a Peltier temperature controller using a bandwidth
177 of 1 nm and a response time of 4 s. Measurements were performed at pH 7.4, in 10 mM
178 phosphate buffer using protein samples at 0.8 DO₂₈₀ (near-UV region) and 0.4 DO₂₈₆
179 (near- and far-UV regions) for amandin, and 0.12 DO₂₈₀ for Ber e 2. Collected spectra
180 were the average of three accumulations and buffer baseline was subtracted. Thermal
181 denaturation was monitored by registering the spectrum as the temperature was raised
182 from 20 to 97 °C for amandin and from 25 to 85 °C for Ber e 2, at a rate of 1 °C min⁻¹,
183 and following the ellipticity change at 283 nm at steps of 0.2 °C. The non-linear fitting
184 of the thermal denaturation profile of amandin to a sigmoidal function was performed

185 using the Origin 8.0 software (OriginLab Corp.). Experiments were performed at the
186 Instituto de Química-Física Rocasolano, CSIC, Madrid (Spain).

187 **RESULTS AND DISCUSSION**

188 Seed storage proteins can represent up to 50 % of the total amount of proteins in nuts.
189 11S globulins (legumin-like proteins), 7S globulins (vicilin-like proteins) and 2S
190 albumins are seed storage proteins able to elicit allergic responses in sensitized
191 individuals.²² In particular, Brazil nut 2S sulphur-rich seed storage albumin (Ber e 1),
192 11S globulin (Ber e 2) and almond 11S globulin (amandin, Pru du 6), are recognized as
193 major allergens by the World Health Organization and International Union of
194 Immunological Societies (WHO/IUIS). Other major tree nuts allergens from the
195 profilin, pathogenesis-related, and IgE binding families have also been described.^{22–26}

196 Phage-scFv clones PD1F6 and PD2C9 detected specifically almond proteins when used
197 in indirect phage-ELISA assays, achieving a detection limit (LOD) of 110-120 mg kg⁻¹,
198 whereas phage-scFv clone BE95 was able to specifically detect Brazil nut proteins with
199 a LOD of 5000 mg kg⁻¹. Furthermore, almond and Brazil nut specific phage scFv were
200 able to detect their target proteins when assaying commercial food products that
201 declared the offending food in the label.^{15,16}

202 In order to characterise the specific protein or proteins recognized by each of the scFv
203 antibodies, a SDS-PAGE in denaturing conditions of almond and Brazil nut protein
204 extracts was performed (**Figure 1A**). In the case of almond, the most prominent bands
205 had a molecular weight of ~40, ~45 kDa, and ~20, ~22 kDa, which according to Sathe
206 et al. (2002) correspond to the complex heterohexameric 11S seed storage protein (also
207 known as amandin or Pru du 6).²⁷ On the other hand, Brazil nut electrophoretic profile

208 exhibited multiple bands, with molecular weights of the most prominent ones being of
209 ~35, ~20 and ~18 kDa. According to Sun et al., 1987, those bands correspond to the
210 11S complex protein fraction (Ber e 2), which possesses six major polypeptides ranging
211 from ~24 to ~32 kDa.²⁸ Sharma et al. (2010) also described Ber e 2 as being composed
212 of two major types of polypeptides: a ~30-32 kDa (acidic subunit) and a ~20-21 kDa
213 (basic subunit), both linked by a disulfide bond.²⁹

214 The ability of phage clones BE95, PD1F6 and PD2C9 to recognize SDS-PAGE
215 separated proteins was assessed by immunoblotting. However, after many attempts of
216 performing the western blotting assay, no binding of the phage scFv to any of the nut
217 proteins was observed under SDS-denaturing conditions. These results support the idea
218 that phage clones express scFv that might recognize conformational epitopes.

219 Recognition of conformational epitopes depends on preservation of tertiary structure of
220 the protein, which is destroyed under the denaturing conditions employed during the
221 SDS-PAGE. However, it is also possible that the phage-scFv used in the present work,
222 selected against a whole protein extract, might recognize targets other than proteins.³⁰⁻³²

223 Hence, electrophoresis and western blotting of Brazil nut and almond whole extracts
224 were performed in non-denaturing conditions, following the procedure described in
225 Ornstein (1964),¹⁷ which employs a discontinuous buffer system. Once the proteins
226 were transferred to the membrane and incubated with the appropriate scFv clone, a
227 smear of chemiluminescence was observed in lanes loaded with almond protein and
228 incubated with phage clones PD1F6 and PD2C9, indicating that those phage clones
229 might recognize proteins products isolated from almond (data not shown). Using the
230 same procedure, no positive signal was obtained for Brazil nut proteins after incubation
231 with BE95 phage clone. A continuous buffer system was then used for the non-

232 denaturing separation of proteins from Brazil nut extract. As the target protein could be
233 affected by pH, gel and running buffer were adjusted to pH 7.4, similar to PBS used to
234 prepare dilutions during the ELISA assay. Using this method, the smeared bands shown
235 in **Figure 1B** were obtained. These results suggested that phage clones might indeed
236 recognize a conformational epitope that suffered denaturation during the SDS-PAGE
237 procedure. In order to confirm whether BE95 phage-scFv clone detected a protein or
238 any other component of the nut, Brazil nut protein extracts were also digested with
239 proteases (trypsin and proteinase K), or thermally treated at 95 °C for 10 min, and
240 extracts were used to coat ELISA plates. No phage binding appeared in either protease
241 treated extracts or boiled extracts assayed by ELISA (**Figure 2**). These results further
242 confirmed that proteins were the main targets of BE95 phage. Although non-denaturing
243 western blotting conditions produced some binding results, the lack of resolution of the
244 technique did not allow the identification of the target proteins.

245 As thermal treatment of protein extracts seemed to affect the ability of phage clones to
246 recognize target proteins, a second approach entailing the repetition of SDS-PAGE
247 under milder conditions (*i.e.* without boiling sample buffer) followed by western
248 blotting analysis was carried out. Under these conditions, almond-phage clones were
249 able to recognize a protein band larger than 150 kDa, which according to the molecular
250 size might correspond to amandin. The same strategy was repeated for Brazil nut
251 protein extract and BE95 phage-clone, without any positive binding results.

252 *Immunoreactivity of Brazil nut protein fractions obtained by size-exclusion* 253 *chromatography*

254 Size-exclusion chromatography of the Brazil nut protein extract resulted in a 280 nm
255 profile with 3 major peaks (A, B, and F) and 4 smaller ones (C, D, E and G) (**Figure**

256 **3A**). ELISA analysis of samples from the eluted fractions showed that phage-scFv
257 antibody BE95 recognizes one of the proteins integrating peaks A and B (data not
258 shown). Therefore, to increase the resolution of chromatographic separation, 1 mL of
259 fraction B was loaded again onto the size-exclusion column. This time, the obtained
260 chromatogram exhibited two separate peaks (**Figure 3A inset**), whose fractions were
261 again subjected to ELISA. **Figure 3B** shows that BE95 clone was able to recognize
262 mainly the components from the first peak, experiencing a gradual decrease in
263 immunoreactivity with components belonging to the second peak. Coomassie staining
264 of the SDS-PAGE gel was not sensitive enough to detect the proteins present in
265 fractions 5 and 10 coming from the second round of size-exclusion chromatography
266 (**Figure 3C**). Nevertheless, the lane loaded with peak B from the first round of size-
267 exclusion chromatography showed 4 major bands, of ~32, ~22, ~20 and ~15 kDa. As
268 the first peak comprises the largest molecular weight proteins, results seem to suggest
269 that Brazil nut phage clones was binding to 11S globulins. 11S storage proteins are
270 complex multimeric proteins, existing as trimers and hexamers, held together by non-
271 covalent interactions that in some seeds represent over 50 % of the total protein.³³ They
272 comprise of acidic (30- 40 kDa) and basic (20 kDa) polypeptide chains linked by a
273 single intermolecular disulfide bond.²⁷ Furthermore, estimated molecular masses of
274 polypeptides comprising 11S globulin from Brazil nut are consistent with earlier
275 findings.^{28,29}

276 *Ion exchange chromatography separation of amandin*

277 In order to confirm whether almond 11S storage protein was indeed the major target for
278 PD1F6 and PD2C9 phage antibodies, an ion exchange procedure for the purification of
279 amandin was carried out, which resulted in a chromatographic profile with a single peak

280 **(Figure 4)**. The purity of the protein was confirmed by SDS-PAGE analysis of peak
281 fractions 3, 5, 7 and 9. In order to prove whether this protein peak was indeed a
282 complex multimeric protein, each fraction was diluted in sample buffer and half of the
283 volume was boiled for 10 min at 95 °C, while the other half was directly loaded onto a
284 gel. **Figure 4 (inset)** shows that in non-heated samples, a large band with a molecular
285 weight higher than 150 kDa, and also two bands somewhat above 30 kDa appeared.
286 These bands were not present when the protein samples were heated. In non-boiled
287 samples, fainter bands with molecular weights around 20kDa and 10-12 kDa were also
288 present, but their intensity greatly increased in heated samples. These results suggest
289 that the bands above 30 kDa are associated forms of the smaller components.
290 Interestingly, as it was evident that a large multimeric protein can be obtained with SDS
291 buffer under milder conditions, the western blotting analysis was then repeated. In those
292 conditions, phage clones PD1F6 (**Figure 5**) and PD2C9 (data not shown) were able to
293 recognize the largest molecular weight protein band from almond total protein extract,
294 and also from the purified amandin.

295 *Analytical Ultracentrifugation*

296 To further characterize the degree of purification and quaternary structure of the
297 amandin isolated by ion exchange chromatography, and the Brazil nut 11S globulin
298 isolated by size exclusion chromatography, sedimentation velocity experiments were
299 performed. On the basis of ultracentrifugation studies, Brazil nut seed storage proteins
300 have been reported to be composed of 11S legumin (also known as excelsin or Ber e 2;
301 the most abundant), 2S albumin and 7S vicilin.²⁸ In almond, four fractions of 2S, 9S,
302 14S and 19S have been identified, being 14S amandin the major component.³⁴ As
303 shown in **Figure 6A**, the distribution of the sedimentation coefficients of amandin

304 displays a single peak with a $s_{20,w}$ of 13.3 S, a value intermediate between those of 13.0
305 and 14 S previously reported for the amandin hexamer.³⁴ In contrast, the globulin
306 isolated from Brazil nut shows a major, symmetric peak (~85%) at 11.95 S (**Figure**
307 **6B**), which agrees with the $s_{20,w}$ values (11.6 - 11.8 S) reported for the hexamer of
308 excelsin,^{35,36} and two minor components at 7.30 S (11.5%) and 4.88 S (3.4%), the
309 former of which might correspond to the trimeric form of excelsin or to a minor fraction
310 of the 7S vicilin, for which a sedimentation coefficient of 7.1 has been reported.²⁹

311 *Peptide identification*

312 To identify almond proteins isolated thus far, 8 electrophoretic bands were excised and
313 trypsinized to be identified by MALDI-TOF/TOF (**Figure 7A**). As shown in **Table 1**,
314 all bands were identified as different isoforms of the 11S seed storage protein (amandin,
315 Pru du 6). Up to date, two isoforms of amandin have been identified: Pru du 6.01
316 (prunin-1, Pru1) and Pru du 6.02 (prunin-2, Pru2). These isoforms share 64% amino
317 acid sequence identity and 77% similarity. Amandin isoforms are each composed of two
318 polypeptides, a large 40 kDa acidic α -chain and a small 20 kDa basic β -chain, which are
319 linked by a disulfide bond. SDS-PAGE analysis of amandin shows the presence of
320 double bands at ~35- 43 kDa, which indicate the presence of different post-
321 translationally processed N-terminal domains. In its native state, amandin is a hexamer,
322 with a molecular weight of 427.3 ± 47.6 kDa.^{27,37,38} Amandin has also shown to be the
323 major allergen recognized by IgE from almond-allergic patients.^{27,39} Interestingly,
324 although the two isoforms have been cloned and expressed, not all sera from allergic
325 patients bind the individual purified isoforms. This observation, and the work carried
326 out with the murine MAb 4C10,³⁸ further stress the importance of conformational
327 epitopes on the allergic sensitisation. It is noteworthy to mention that ion-exchange

328 chromatography purified amandin showed a smaller molecular weight band when
329 compared with amandin from the complete extract, which was also analysed to verify
330 protein identity (**Figure 5, Table 1**). As it is observed in **Figure 7A**, basic β -chain
331 molecular weight from purified amandin is in accordance with the same protein from
332 the complete almond extract (20 kDa). However, there is a disagreement between acidic
333 α -chain peptide bands from purified amandin and complete almond extract. Therefore,
334 the difference in molecular weight that exhibited the largest band of amandin in SDS-
335 PAGE must be due to a cleavage suffered by the acidic α -chain peptide.

336 On the other hand, SDS-PAGE electrophoresis of fraction B from size-exclusion
337 chromatography of Brazil nut protein extract (**Figure 7B**) revealed 4 bands that were
338 excised and trypsinized to perform a MALDI-TOF/TOF analysis. Furthermore, a
339 sample from fraction 5 of the size exclusion fractionation of peak B was also analysed.
340 **Table 2** shows that all electrophoretic bands, as well as peak 5 liquid sample, were
341 identified as 11S globulin from Brazil nut. However, theoretical tryptic cleavage had to
342 be done employing the sole database entry existing for 11S globulin from Brazil nut
343 (gi|30313867). Despite showing high sequence coverage and ion score values
344 confirmation, there was no possible way to elucidate if the sample analyzed contained
345 more than one isoform for 11S globulin. Previous studies have reported that purified
346 11S globulins from Brazil nut contain multiple bands on SDS-PAGE, and that the
347 protein is post-translationally cleaved, yielding an N-terminal acidic subunit and a C-
348 terminal basic subunit linked by an interchain disulfide bond, as 11S globulins from
349 different species.^{33,37,40}

350 *Circular dichroism analysis and thermal stability of target proteins*

351 ELISA and western blotting analysis of purified amandin and Brazil nut 11S globulin
352 demonstrated that Brazil nut specific BE95 phage-scFv, and almond specific PD1F6
353 phage-scFv were unable to recognize their target proteins following heat denaturation.
354 In order to understand the thermal stability of amandin and Brazil nut 11S globulin,
355 changes in ellipticity of these proteins as a function of temperature were followed and
356 monitored by CD. The Far-UV CD spectrum of amandin at 20 °C displayed two minima
357 around 209 and 224 nm and remain nearly unchanged from 20 to 80 °C (**Figure 8A**), in
358 agreement with previous observations.⁴¹ Thermally induced changes in this region of
359 the spectra are moderate above 209 nm, which hamper to follow amandin denaturation.
360 The loss of tertiary and quaternary structure was therefore monitored by following the
361 ellipticity variation at the near-UV region of the CD spectrum where amandin displays a
362 maximum at 284 nm (**Figure 8B**). The signal disappears between 75 and 97 °C (**Figure**
363 **8C**) but a reliable estimation of the half transition temperature is precluded by the
364 absence of the post-transition region. When involved in tertiary/quaternary interaction
365 network, phenylalanines generally absorb in the 258-268 nm, tyrosines in the 274-286
366 nm and triptophanes in 260-300 nm.⁴² In the case of Ber e 2, the far-UV CD spectrum at
367 25 °C revealed two minima at around 208 and 216 nm (**Figure 8D**), in agreement with
368 the spectrum reported for this protein.²⁹ Furthermore, changes in ellipticity, monitored
369 by CD, revealed that no major changes in the secondary structure were observed within
370 the 25-75 °C range. Nevertheless, when temperature reached 85 °C, the intensity of
371 minima decreased and the major minimum slightly blue-shifted. The limited
372 concentration of Brazil nut 11S protein available (37.25 µg mL⁻¹) avoided further
373 studying its thermal stability.

374 These results explain the lack of immunoreactivity in Western-blot analysis of amandin
375 boiled in sample buffer before electrophoretic separation, but not in the absence of heat
376 treatment. The negative results obtained with the phage-scFvs in ELISA after boiling
377 the nut extracts can be also explained by the lack of thermal stability of the target
378 proteins at temperatures higher than 85 °C.

379 Nevertheless, absence of target recognition after boiling protein extracts contrasted with
380 the ability of the same phage-scFv to recognize the target protein when ground nuts
381 underwent severe heat treatments (roasting at 160 °C for 13 min, or autoclaving for 15
382 min at 121 °C), previously to the preparation of the protein extracts, as described
383 before.¹⁵⁻¹⁶ In this sense, Mills et al. (2003), stated that proteins show an increase in
384 thermostability when encountered in low-water systems, such as whole food matrices.³³
385 Therefore, it can be argued that heating proteins in solution might enhance denaturation
386 of proteins, which would explain why phage-scFv were still able to detect their target
387 proteins in processed foods when analysed by ELISA.

388 In summary, ion exchange chromatography, mass spectrometry (MS) and western
389 blotting analysis results supported that PD1F6 and PD2C9 phage-scFv clones, isolated
390 against a complete almond protein extract, recognized a conformational epitope of
391 almond 11S globulin legumin-like protein, also named amandin, which corresponds to
392 the almond major allergen Pru du 6. On the other hand, size exclusion fractionation and
393 MS results revealed that BE95 phage-scFv clone, isolated against a whole Brazil nut
394 protein extract, recognized a conformational epitope of the Brazil nut 11S globulin,
395 corresponding to allergen Ber e 2. Performance of phage library screening procedure
396 using a complete protein extract is consistent with the isolation of phage-scFv targeting
397 the major seed storage allergenic proteins contained in the extract. Lack of recognition

398 of the target proteins observed after severe heat treatments of protein extracts was in
399 accordance with the results obtained in CD.

400 The results obtained in this work revealed that isolated phage-displayed scFv target the
401 native forms of two major allergens from Brazil nut and almond, and they would serve
402 as a useful tool to detect the presence of allergenic tree nuts in foodstuffs.

403 **ABBREVIATIONS USED**

404 BCA, bicinchoninic acid assay ; BSA, bovine serum albumin; CD, circular dichroism;
405 ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid
406 chromatography; IgE: type E immunoglobulin; MALDI, matrix-assisted laser
407 desorption/ionization; MS, mass spectrometry; PAGE, polyacrylamide gel
408 electrophoresis; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride;
409 TOF, time of flight.

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543

544 **Table 1:** Almond peptides identified by MALDI-TOF/TOF Tandem Mass Spectrometry
 545 and Mascot Database Search.

Gel band	Protein identification	Accession number	Sequence coverage	Total score	Ion scores	Peptide sequences
1	prunin 1 precursor [<i>Prunus dulcis</i>] prunin 2 precursor, partial [<i>Prunus dulcis</i>]	gi 307159112 gi 307159114	35% 37%	380 285	80 136 87 65 20 104	R.ISTLNSHNLPILR.F R.ALPDEVLANAYQISR.E K.YNRQETIALSSSQQR.R R.ADFYNPQGGR.I R.EGQLFLIPQNHAVITQASNEGFEYISFR.T R.ALPDEVLQNAFR.I
2	prunin 1 precursor [<i>Prunus dulcis</i>]	gi 307159112	31%	182	123	R.ALPDEVLANAYQISR.E
3	Chain A, Crystal Structure Of Pru Du Amandin, An Allergenic Protein From <i>Prunus dulcis</i>	gi 258588247	44%	650	46 89 131 139 96	R.GVLGAVFSGCPETFEESQQSSQGR.Q R.ISTLNSHNLPILR.F K.TEENAFINTLAGR.T R.ALPDEVLANAYQISR.E K.YNRQETIALSSSQQR.R
4	prunin [<i>Prunus dulcis</i>]	gi 460806	29%	451	80 135 98 53	R.ISTLNSHNLPILR.F R.ALPDEVLANAYQISR.E K.YNRQETIALSSSQQR.R R.QETIALSSSQQR.R
5	prunin 2 precursor, partial [<i>Prunus dulcis</i>]	gi 307159114	28%	322	33 84 16 112	R.LSQNIGDPSRADFYNPQGGR.I R.ADFYNPQGGR.I R.EGQLFLIPQNHAVITQASNEGFEYISFR.T R.ALPDEVLQNAFR.I

6	prunin 1 precursor [Prunus dulcis]	gi 307159112	7%	87	52 21	R.GNLDFVQPPR.G R.QQEQLQQR.Q
7	prunin 1 precursor [Prunus dulcis]	gi 307159112	27%	279	74 58 85	R.QSQLSPQNQCQLNQLQAR.E R.GVLGAVFSGCPETFEESQQSSQQ GR.Q R.KFYLAGNPENEFNQQGQSQPR.Q
8	prunin 1 precursor [Prunus dulcis]	gi 307159112	25%	197	15 28 56 45	R.IQAEAGQIETWNFNQEDFQCAGV AASR.I R.GVLGAVFSGCPETFEESQQSSQQ GR.Q R.KFYLAGNPENEFNQQGQSQPR.Q R.GNLDFVQPPR.G
*	Idem bands 1, 3 and 5 Idem band 1, 2, 6, 7, 8 pru2, partial [Prunus dulcis]	gi 258588247 + gi 307159114 gi 307159112 + gi 460816	31% 47%	146 195		

546

547 **Table 2:** Brazil nut peptides identified by MALDI-TOF/TOF Tandem Mass

548 Spectrometry and Mascot Database Search.

Gel band	Protein identification	Accession number	Sequence coverage	Total score	Ion scores	Peptide sequences
9	11S globulin [<i>Bertholletia excelsa</i>]	gi 30313867	23%	92	33 31	R.LEAEAGVSEVWDYTDQQFR.C R.NTIRPQGLLLPVYTNAPK.L
10	11S globulin [<i>Bertholletia excelsa</i>]	gi 30313867	32%	116	28	R.GETVFDNLR.E
11	11S globulin [<i>Bertholletia excelsa</i>]	gi 30313867	33%	188	58 51	K.GVLYENAMMAPLWR.L K.LNRDEAVLFQPGSR.S
12	11S globulin [<i>Bertholletia excelsa</i>]	gi 30313867	2%	48	48	R.HFFLAGNIQR.S
Peak 5	11S globulin [<i>Bertholletia excelsa</i>]	gi 30313867	9%	199	83 36 90	S.EVWDYTDQQFR.C R.NTIRPQGLLLPVYTNAPK.L T.FIQNIDNPAEADFYNPR.A

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