TITLE

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.

INTRODUCTION

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

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

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

display antibody fragments fused to one of the phage coat proteins have been successfully used for this purpose.[13,14]

In two previous works,[15,16] we reported the isolation of a specific scFv against Brazil nut (named BE95) and two specific scFv against almond (named PD1F6 and PD2C9) from the commercial phage library Tomlinson I, using crude protein extracts from Brazil nut or almond as targets to perform the selection procedure. Those phage-clones were successfully used in indirect phage-ELISA to detect the presence of Brazil nut and almond in commercial food products. However, as library screenings were performed employing a whole nut protein extract, the identity of the targets still remains unknown. Hence, the main objective of this work was to identify and characterise the specific protein or proteins from the tree nut extracts detected by each of the scFv antibodies.

MATERIALS AND METHODS

Materials and Chemicals

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

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

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

Preparation of protein extracts

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

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

SDS-PAGE and western blotting analysis

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

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

Size-exclusion chromatography

Size-exclusion chromatography separation was carried out in a fast protein liquid chromatography (FPLC) system (Pharmacia-LKB, Uppsala, Sweden). Protein extract (1 mL) of Brazil nut, prepared as described before, was injected into a HiPrep 16/60 Sephacryl S-200 HR column (GE, Healthcarre UK Ltd., Buckinghamshire, UK) 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 ºC 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 4º C. Next day,
124 plates were washed 3 times, and blocked with 1% ovalbumin in PBS for 1 h at 37 ºC.
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. OD_{450} 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 H_2O (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

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

Analytical Ultracentrifugation

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

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

Peptide identification

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

Circular dichroism

Circular dichroism (CD) spectra were recorded with a Jasco-810 spectropolarimeter (JASCO Ltd, Spain) equipped with a Peltier temperature controller using a bandwidth of 1 nm and a response time of 4 s. Measurements were performed at pH 7.4, in 10 mM phosphate buffer using protein samples at 0.8 DO_{280} (near-UV region) and 0.4 DO_{286} (near- and far-UV regions) for amandin, and 0.12 DO_{280} for Ber e 2. Collected spectra were the average of three accumulations and buffer baseline was subtracted. Thermal denaturation was monitored by registering the spectrum as the temperature was raised from 20 to 97 ºC for amandin and from 25 to 85 ºC for Ber e 2, at a rate of 1 ºC min^{-1}, and following the ellipticity change at 283 nm at steps of 0.2 °C. The non-linear fitting 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

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

The ability of phage clones BE95, PD1F6 and PD2C9 to recognize SDS-PAGE separated proteins was assessed by immunoblotting. However, after many attempts of performing the western blotting assay, no binding of the phage scFv to any of the nut proteins was observed under SDS-denaturing conditions. These results support the idea that phage clones express scFv that might recognize conformational epitopes. Recognition of conformational epitopes depends on preservation of tertiary structure of the protein, which is destroyed under the denaturing conditions employed during the SDS-PAGE. However, it is also possible that the phage-scFv used in the present work, selected against a whole protein extract, might recognize targets other than proteins.[30–32] Hence, electrophoresis and western blotting of Brazil nut and almond whole extracts were performed in non-denaturing conditions, following the procedure described in Ornstein (1964),[17] which employs a discontinuous buffer system. Once the proteins were transferred to the membrane and incubated with the appropriate scFv clone, a smear of chemiluminescence was observed in lanes loaded with almond protein and incubated with phage clones PD1F6 and PD2C9, indicating that those phage clones might recognize proteins products isolated from almond (data not shown). Using the same procedure, no positive signal was obtained for Brazil nut proteins after incubation with BE95 phage clone. A continuous buffer system was then used for the non-

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

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

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

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

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

Ion exchange chromatography separation of amandin

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

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

Analytical Ultracentrifugation

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

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

Peptide identification

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

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

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

Circular dichroism analysis and thermal stability of target proteins

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

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

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

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

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

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

ABBREVIATIONS USED

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

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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 [*Prunus dulcis*] prunin 2 precursor, partial [*Prunus dulcis*]	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 [*Prunus dulcis*]	gi|307159112	31%	182	123	R.ALPDEVLANAYQISR.E
3	Chain A, Crystal Structure Of Pru Du Amandin, An Allergenic Protein From *Prunus dulcis*	gi|258588247	44%	650	46 89 131 139 96	R.GVLGAVFSGCPETFEESQQSSQQGR.Q R.ISTLNSHNLPILR.F K.TEENAFINTLAGR.T R.ALPDEVLANAYQISR.E K.YNRQETIALSSSQQR.R
4	prunin [*Prunus dulcis*]	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 [*Prunus dulcis*]	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.QQEQLQQER.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		

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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 [*Bertholletia excelsa*]	gi|30313867	23%	92	33 31	R.LEAEAGVSEVWDYTDQQFR.C R.NTIRPQGLLLPVYTNAPK.L
10	11S globulin [*Bertholletia excelsa*]	gi|30313867	32%	116	28	R.GETVFDDNLR.E
11	11S globulin [*Bertholletia excelsa*]	gi|30313867	33%	188	58 51	K.GVLYENAMMAPLWR.L K.LNRDEAVLFQPGSR.S
12	11S globulin [*Bertholletia excelsa*]	gi|30313867	2%	48	48	R.HFFLAGNIQR.S
Peak 5	11S globulin [*Bertholletia excelsa*]	gi|30313867	9%	199	83 36 90	S.EVWDYTDQQFR.C R.NTIRPQGLLLPVYTNAPK.L T.FIQNIDNPAEADFYNPR.A

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