

Short communication

Epitope mapping of the major allergen 2S albumin from pine nut

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ABSTRACT

The epitopes of the major allergen of pine nut, Pin p 1, were analyzed using a peptide library and sera from patients with clinical allergy to pine nut in order to deepen into the allergenic characteristics of Pin p 1. Analyses of epitope similarities and epitopes location in a 3D-model were also performed. Results showed that three main regions of Pin p 1 containing 5 epitopes were recognized by patient sera IgE. The epitopes of Pin p 1 had important similarities with epitopes of allergenic 2S albumins from peanut (Ara h 2 and 6) and Brazil nut (Ber e 1). The epitopes of Pin p 1 were found in α -helices and coils in the 3D protein structure. Interestingly, all epitopes were found to be well-exposed in the protein surface, which suggests facile access for IgE-binding to the structure of Pin p 1 which is known to be highly resistant.

1. Introduction

Allergy to pine nut has been described to be particularly severe and to be characterized by a high rate of monosensitized patients (Cabanillas, Crespo, Maleki, Rodriguez, & Novak, 2016; Cabanillas et al., 2012). The first allergen of pine nut from *Pinus pinea* L., the largest producer species of pine nut in Europe, has been recently characterized as a 2S albumin and named as Pin p 1 by the World Health Organization and International Union of Immunological Societies (WHO/IUIS). 2S albumins are seed storage protein belonging to the prolamin superfamily and are commonly characterized by a compact 3D structure with conserved helical regions that provides them with high resistance to pH, enzymes, and heat. These characteristics allow 2S albumins to keep their structure intact until they are absorbed in the gut and reach the effector cells of the immune system (Moreno & Clemente, 2008). 2S albumins have been described as major allergens in nuts such as peanut (Ara h 2, 6, and 7), Brazil nut (Ber e 1), hazelnut (Cor a 14), pistachio (Pis v 1) or cashew (Ana o 3) (Cabanillas et al., 2016). Pin p 1 was described as a major allergen in pine nut, and similarly to other 2S albumins, it was highly resistant to heat and gastrointestinal enzymes and with a robust capacity to crosslink IgE on basophils (Cabanillas et al., 2016, 2012). Pin p 1 had a compact structure with a disulfide-linked conserved 3D motif similar to other allergenic 2S albumins from

more evolved species producers of tree nuts such as hazelnut, walnut, cashew, or seeds such as mustard seeds. However, the amino acid sequence of Pin p 1 was found to have a significant low similarity with other allergenic 2S albumins. The marked evolutionary differences of Pin p 1 with the rest of allergenic 2S albumins have been pointed out as a relevant factor in the differential features of pine nut allergy (Bueno, Martín-Pedraza, Cuesta-Herranz, & Villalba, 2016). Yet, analyses on the immunodominant IgE-binding regions of Pin p 1 and their comparison with those of other allergenic 2S albumins, that would allow us to understand such differences, are lacking. We hypothesized that Pin p 1 may contain relevant immunodominant regions that bind IgE which could explain the allergenic potential of this major allergen from pine nut. In order to test this hypothesis, in this study, an analysis of the IgE-binding epitopes of Pin p 1 using sera from patients with well-characterized clinical allergy to pine nut was carried out in order to deepen into the allergenic characteristics of Pin p 1 and to study its differential features with other 2S albumins.

2. Material and methods

2.1. Patients

Sera from eight patients diagnosed with IgE-mediated clinical

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Table 1
Characteristics of the patients with pine nut allergy included in this study.

Patient No.	Age	Sex	SPT (mm)	CAP-FEIA (kU/L)	Symptoms	Diagnostic challenge
P1	38	F	11.5	0.9	U, AE, P, OAS, DS, TS, A, GM	a
P2	26	M	17.5	48	U	a
P3	34	M	12	2.27	AE, P, E, OAS, GI, DS, TS, GM	a
P4	18	F	15.5	2.2	AE, DY, DS, OAS, GI	a
P5	17	M	5.5	19.3	OAS	DBPCFC
P6	35	F	11	7.08	U, P, OAS, GI, GM	a
P7	30	F	4.5	0.8	OAS	DBPCFC
P8	39	F	10	0.02	AE, P, E, OAS, O, TS	a

AE, angioedema; A, asthma; CAP-FEIA, capsulated hydrolytic carrier polymer-fluoroenzyme immunoassay; DBPCFC, double-blind, placebo-controlled food challenge; DS, difficulty of swallowing; DY, dysphonia; E, erythema; GI, gastrointestinal symptoms; GM, general malaise; OAS, oral allergy syndrome; O, ocular symptoms; P, pruritus; SPT, skin prick testing; TS, tongue swelling; U, urticaria. a, challenge not performed because of a history of severe reactions to pine nut.

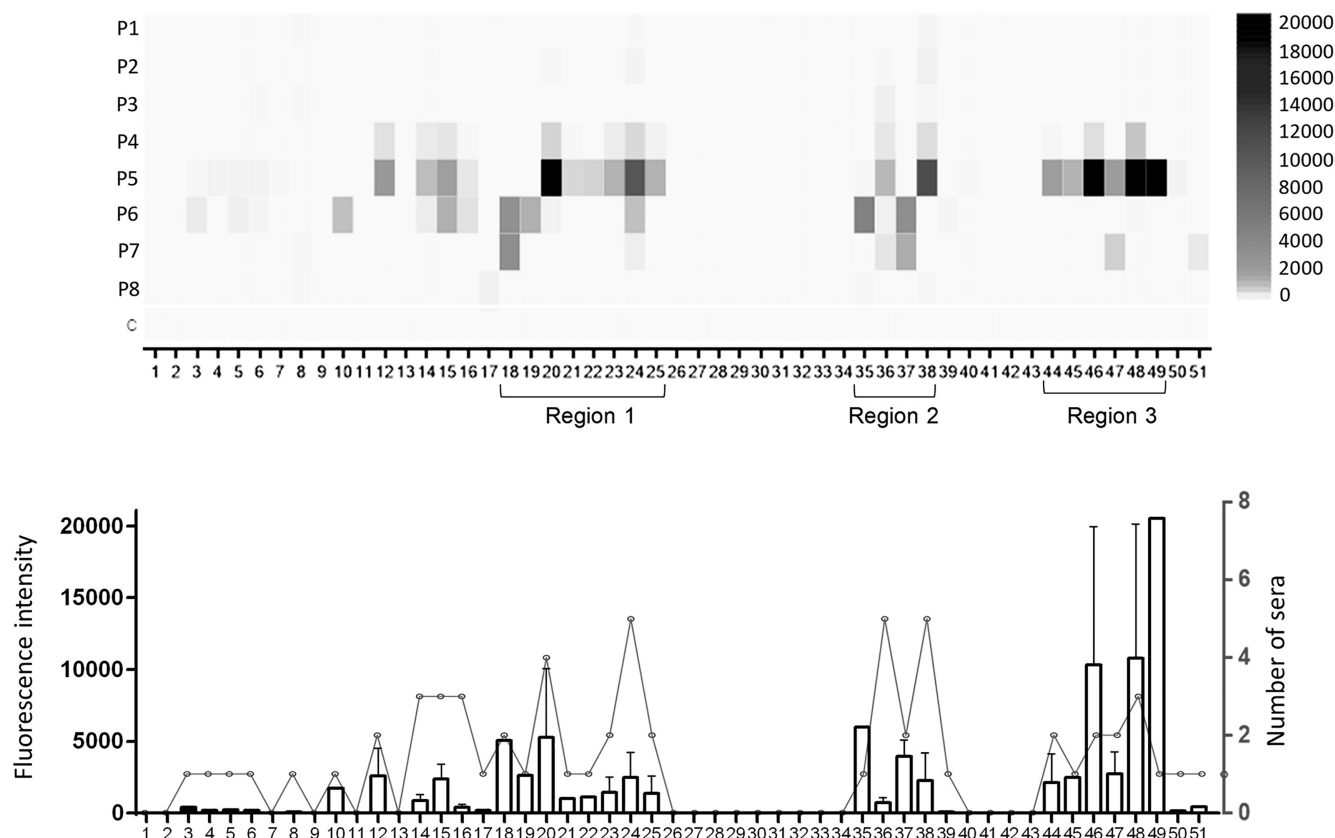


Fig. 1. IgE-recognition by individual sera (P1-P8) (C: control serum) of the peptide library covering the complete sequence of Pin p 1 (51 peptides of 15 amino acids length with an overlap of 12 amino acids). The upper part of Fig. 1A shows a heatmap diagram displaying fluorescence intensities of each peptide recognized by serum IgE. The lower part of Fig. 1 shows a bar-line graph representing the mean fluorescence intensities of the peptides with positive values (bars) (left y-axis), and the number of sera that recognizes each peptide (lines) (right y-axis).

allergy to pine nut were used in this study. The diagnosis of pine nut allergy in these patients was established by either a history of recent and documented severe anaphylaxis after the ingestion of pine nuts or by a positive double-blind placebo-controlled food challenge (DBPCFC) with pine nuts. All patients had specific IgE against pine nut (CAP-FEIA system, Phadia, Uppsala, Sweden) and/or positive skin prick test to peanut (prick-to-prick technique using standard methods, Heinzerling et al., 2013) (Table 1). Written informed consents were obtained from all patients. The study was approved by the Ethics Committee of the Hospital Universitario 12 de Octubre, Madrid, Spain (Permission No. 0312150129).

2.2. Peptide library of Pin p 1 in glass microarrays slides and detection of specific IgE against Pin p 1 peptides.

A library of 51 peptides consisting on 15-meric peptides, with an overlap of 12 amino acids, covering the complete sequence of Pin p 1 previously described (Cabanillas et al., 2016) was synthesized, purified, and covalently immobilized into glass microarray slides using PepStar technology (JPT Peptide Technologies, Berlin, Germany). The peptide library was printed in triplicate for incubation with individual sera from eight patients diagnosed with IgE-mediated clinical allergy to pine nut. Serum from a subject without pine nut allergy was also included as a control. Individual sera samples were diluted at 1:5 in blocking buffer (Superblock TBS T20, ThermoFisher Scientific, San Jose, CA, USA) and incubated for 1 h at 30 °C on the microarray slides. After washing the slides with washing buffer (50 mM TBS-buffer including 0.1%

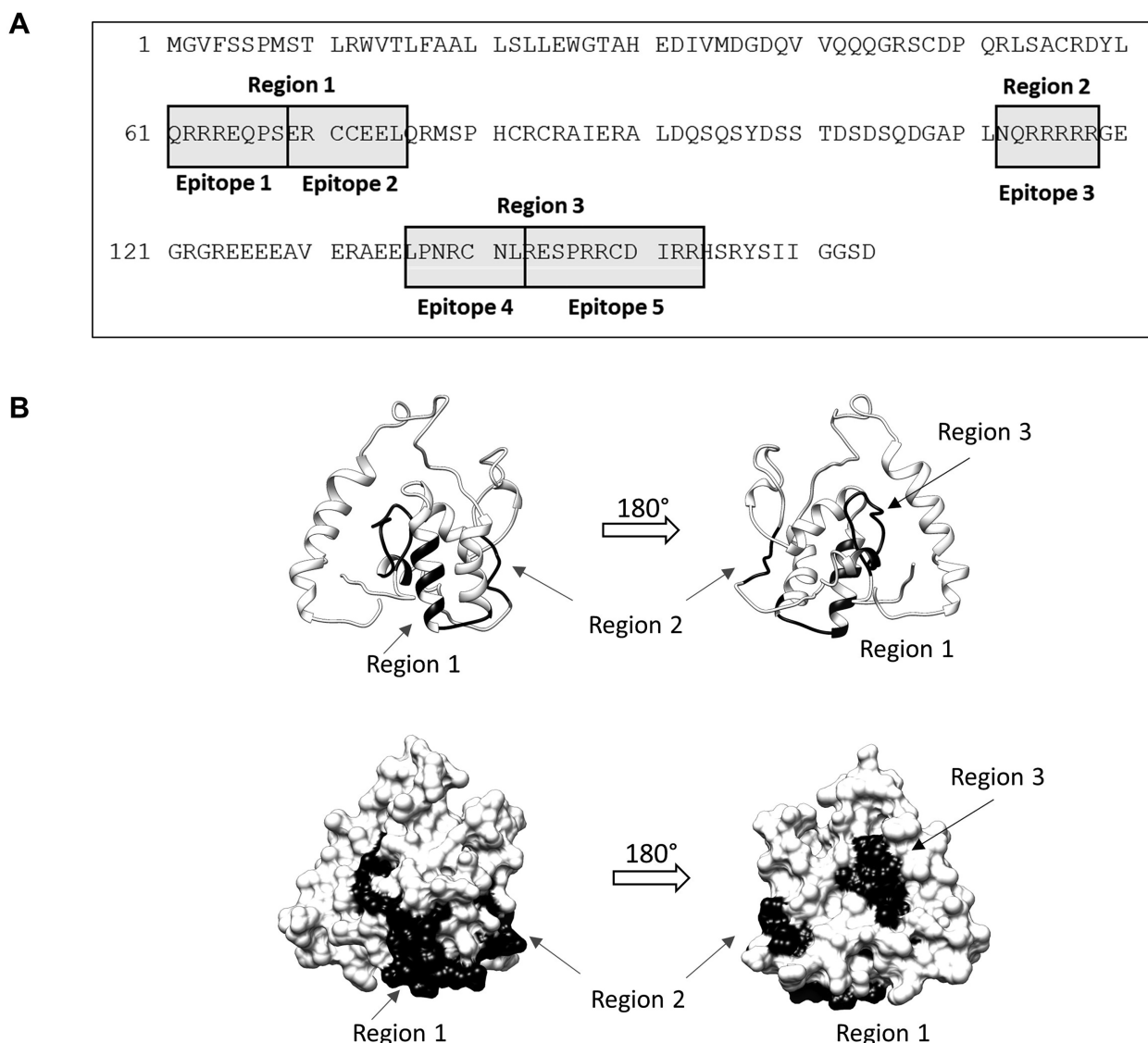


Fig. 2. A. Amino acid sequence of the complete sequence of Pin p 1 in which the 3 main regions containing the 5 IgE-binding epitopes are marked. B. 3D protein prediction model of Pin p 1 and IgE-binding epitopes location. The three regions containing the IgE-binding epitopes of Pin p 1 are indicated in black color in the secondary structure of the protein (upper part of Fig. 2B) and in its surface (lower part of Fig. 2B).

Tween20, pH 7.2), a mouse anti-human IgE secondary antibody (clone GE-1, Sigma-Aldrich, St. Louis, MO, USA) diluted at 0.1 µg/ml in blocking buffer was added and incubated for 45 min at 30 °C. Finally, after washing the slides, a goat anti-mouse IgG tertiary antibody labeled with DyLight 650 (ThermoFisher Scientific, San Jose, CA, USA) diluted in blocking buffer at 0.1 µg/ml was added and incubated for 45 min at 30 °C. As a control, an incubation with the secondary and tertiary antibodies (without sera incubation) was included. After a washing and drying step, the slides were scanned using a high-resolution laser scanner (Axon Genepix Scanner 4300 SL50, Molecular Devices, San Jose, CA, USA) at a wavelength of 635 nm in order to obtain the profile of fluorescence intensities. The settings of the laser and the resolution were equal for all the measurements. The images were quantified using spot-recognition software GenePix (Molecular Devices, San Jose, CA, USA) in order to obtain the mean fluorescence intensity for each spot. The criterium to define an IgE-binding epitope was based on the recognition of a sequence between 6 and 12 amino acids present in 3, 4, or 5 contiguous peptides with a mean fluorescence intensity ≥ 700.5

2.3. Heatmap diagram, study of similarities, and 3D model of Pin p 1

A heatmap diagram displaying fluorescence intensities was created with the obtained results using Displayr software for their visualization and comparison. Similarities of IgE-binding epitopes of Pin p 1 with other peptides from allergens were analyzed using the Structural Database of Allergenic Proteins (SDAP) (Ivanciuc, Schein, & Braun, 2003, 2002). SDAP calculates the PD similarity index between the query sequence and all the allergenic sequences with the same length in the SDAP allergen database. PD values can range from 0 which means exact match between the sequences and a PD value between 7.5 and 9 which has been established as a cut-off positivity value for peptides similarities (Ivanciuc et al., 2003). The alignments of Pin p 1 epitopes with similar peptide sequences from other allergens were performed using Clustal Omega (Sievers et al., 2011). A 3D model of Pin p 1 was generated using Protein Homology/analogy Recognition Engine (PHYRE) (Kelley & Sternberg, 2009) and analyzed with UCSF Chimera (Pettersen et al., 2004) in order to spot the IgE-binding epitopes on the 3D structure of the protein.

A	Region	Epitope	Sequence	Allergens with peptide similarities (PD value)
	Region 1	Epitope 1	QRRREQPS	Cor a 9 (5.59)
		Epitope 2	ERCCEEL	Ara h 2 (2.09); Hor v 1 (2.31); Ara h 6 (2.96); Tri a 29 (3.14); Ara h 7 (3.57)
	Region 2	Epitope 3	NQRRRR	---
	Region 3	Epitope 4	LPNRCNL	Cor a 14 (3.27); Ber e 1 (2.22); Ara h 2 (4.09); Ara h 6 (3.84); Ses i 1 (3.66)
		Epitope 5	RESPRRCDIRR	Cor a 14 (5.54)

B	Epitope 1	Epitope 4
	Pin p 1 QRRREQPS Cor a 9 QRRRQQQH § (Robotham et al., 2009) ****.*	Pin p 1 LPNRCNL Cor a 14 LPNQCRLL ***.*.*
	Epitope 2	Pin p 1 LPNRCNL Ber e 1 IPSRCNL § (Alcocer et al., 2004) *.****
	Pin p 1 ERCCEEL Ara h 2.0101 ERCCNEL § (Otsu et al., 2015) Ara h 2.0201 ERCCNEL § (Otsu et al., 2015) Hor v 1 ERCCREL Ara h 6 QRCCDEL § (Otsu et al., 2015) Ara h 7.0101 QRCCNEL Ara h 7.0201 QRCCNEL Ara h 7.0301 QRCCNEL Tri a 29.0101 DRCCNEL Tri a 29.0201 DRCCNEL :******	Pin p 1 LPNRCNL Ara h 2 LPQQCGL § (Otsu et al., 2015) Ara h 6 LPQQCNF § (Otsu et al., 2015) **.*.*:
		Epitope 5
		Pin p 1 RESPRRCDIRR Cor a 14 --SPQRCEIRS **.*.*

Fig. 3. A. Amino acid sequences of the IgE-binding epitopes of Pin p 1 and PD similarity indexes (PD values; displayed in brackets) between them and peptides from other allergens analyzed by the Structural Database of Allergenic Proteins (SDAP). B. Alignments of the IgE-binding epitopes of Pin p 1 with the peptides from other allergens mentioned in Fig. 3A using Clustal Omega. An asterisk (*) indicates positions with an identical amino acid. A colon (:) indicates a position with amino acids of strongly similar properties. A period (.) indicates a position with amino acids with weakly similar properties. A blank indicates no similarities between amino acids in that position. The symbol § indicates the peptides described as IgE-binding epitopes, or part of IgE-binding epitopes, in the scientific literature (the corresponding reference is specified in brackets). Ara h, *Arachis hypogaea* L. (peanut); Ber e, *Bertholletia excelsa* HBK (Brazil nut); Cor a, *Corylus avellane* L. (hazelnut); Hor v, *Hordeum vulgare* L. (barley); Pin p, *Pinus pinea* L. (pine nut); Ses i, *Sesamum indicum* L. (sesame); Tri a, *Triticum aestivum* L. (wheat).

3. Results and discussion

In this study, the main IgE-binding epitopes of the major allergen from pine nut, Pin p 1, were planned to be characterized and analyzed. For that, a library of overlapping peptides covering the complete sequence of Pin p 1 was utilized. The peptide library was incubated with individual sera from eight patients diagnosed with IgE-mediated clinical allergy to pine (Table 1) and IgE-binding was detected with specific antibodies. Fluorescence intensities analysis, a study of similarities, as well as an epitope mapping in a 3D model of Pin p 1, were carried out.

The results showed that three main regions of the sequence of Pin p 1 were principally recognized by sera IgE from the pine nut allergy patients (Fig. 1). The regions comprise peptides 18–25 (region 1); peptides 35–38 (region 2); and peptides 44–49 (region 3) (Fig. 1). The sequences of the IgE-binding epitopes contained in those regions can be visualized in Fig. 2A and they were: QRRREQPS (epitope 1) and ERCCEEL (epitope 2) for region 1, NQRRRR (epitope 3) for region 2, LPNRCNL (epitope 4) and RESPRRCDIRR (epitope 5) for region 3 (Fig. 2A). Negative results were obtained with the control serum. A predictive 3D model of Pin p 1 was built in order to spot the IgE-binding epitopes on the protein structure. Fig. 2B shows the 3D protein prediction model of Pin p 1 and the location of the IgE-binding epitopes. The results showed that region 1 containing epitopes 1 and 2 was located in an α -helix in the 3D structure, region 2 was part of a coil, and region 3 containing epitopes 4 and 5 was located mainly in a coil (Fig. 2B, upper part). All three regions containing the IgE-binding epitopes of Pin p 1 were well-exposed in the surface of the protein structure (Fig. 2B, lower part). Other studies have also demonstrated that the IgE-binding epitopes of allergens from different sources are usually surface-exposed on the structure of the allergenic protein (Lee, Yoo, Kim, Chung, Cho, & Ju, 2018; Pang et al., 2019).

In another set of analyses, the similarity of the IgE-binding epitopes of Pin p 1 with those of other allergenic peptides was investigated and the PD similarities indexes were calculated as described in material and methods. Fig. 3A shows the PD values of the IgE-binding epitopes of Pin p 1 and these regions from other allergens. Fig. 3B shows the alignments among them. The results showed that the epitope 1 (region 1) of Pin p 1 had similarities with a peptide from Cor a 9, the 11S globulin allergen from hazelnut, which has been described to be part of an epitope-bearing peptide (Robotham et al., 2009) (Fig. 3A, 3B). Furthermore, the epitope 2 of Pin p 1 showed important similarities with peptides of multiple allergenic 2S albumins such as Ara h 2, Hor v 1, Ara h 6, Ara h 7, and Tri a 29 (Fig. 3A, 3B). The IgE-binding epitopes of Hor v 1 (2S albumin allergen from barley) and Tri a 29 (2S albumin allergen from wheat) have not been described so far. However, the IgE-binding epitopes of Ara h 2 and Ara h 6, which are 2S albumin allergens from peanut, have been greatly studied and one of the described epitopes of both allergens showed relevant similarities in part of their sequence with the epitope 2 of Pin p 1 (Otsu, Guo, & Dreskin, 2015) (Fig. 3B). Lower similarity was found for epitope 2 with other 2S albumins such as Pis v 1 (4.37), Sin a 1 (4.81), or Ana o 3 (6.11) (data not shown). The region 3 of Pin p 1, containing epitopes 4 and 5, showed important similarities with two peptides of Cor a 14, the 2S albumin allergen from hazelnut (Fig. 3A, 3B). The IgE-binding epitopes from Cor a 14 have not been analyzed so far, therefore the similarities found in this study could indicate potential IgE-binding epitopes of Cor a 14 in that region. Furthermore, the epitope 4 of Pin p 1 also showed high similarity with a peptide of Ber e 1, the 2S albumin allergen from Brazil nut, which has been described to be part of a major structural IgE epitope of Ber e 1 (Alcocer et al., 2004). Similarities were also found in epitope 4 of Pin p 1 with an IgE-binding epitope of Ara h 2 and Ara h 6 (Otsu, Guo, & Dreskin, 2015) (Fig. 3A, 3B).

Although the clinical relevance of the IgE cross-reactivity between pine nut and other nuts and tree nuts has not been demonstrated so far (Cabanillas & Novak, 2015; Cabanillas et al., 2012), the similarities found in this study between certain IgE-binding epitopes of Pin p 1 with those from highly allergenic nuts such as peanut, Brazil nut or hazelnut suggest that IgE cross-reactivity could be possible between pine nut and other nuts / tree nuts despite their evolutionary distance (Cabanillas et al., 2016).

4. Conclusion

Given the relevance of 2S albumins as potential elicitors of severe allergic reactions (Moreno & Clemente, 2008; Kukkonen, Pelkonen, Mäkinen-Kiljunen, Voutilainen, & Mäkelä, 2015; Blazowski, Majak, Kurzawa, Kuna, & Jerzynska, 2019), their in-depth study at the epitope level constitutes an important tool for the analysis of their allergenic impact. In this study, we have characterized three main regions of Pin p 1 recognized by sera IgE from patients with well-characterized pine nut allergy and we have shown that although Pin p 1 was previously described to have low similarity with other 2S albumins, its IgE-binding epitopes share important similarities with epitopes of allergens such as Ara h 2 and Ara h 6 from peanut or Ber e 1 from Brazil nut. Importantly, Pin p 1 epitopes were found to be well-exposed in the protein surface, which suggests facile access for IgE-binding to the structure of Pin p 1 which is known to be highly resistant to heat and enzymes (Cabanillas et al., 2016, 2012).

Our study contributes to the growing area of research that is focusing on the characterization of allergens' epitopes which will potentially allow going one step forward in the knowledge of allergens for their use in the molecular diagnosis of nut allergy based on individual allergens.

CRedit authorship contribution statement

Jesus F. Crespo: Resources, Investigation, Writing - review & editing. **Cristina Bueno:** Investigation, Writing - review & editing. **Mayte Villalba:** Investigation, Writing - review & editing. **Linda Monaci:** Writing - review & editing. **Carmen Cuadrado:** Investigation, Writing - review & editing. **Natalija Novak:** Conceptualization, Investigation, Writing - review & editing. **Beatriz Cabanillas:** Conceptualization, Investigation, Methodology, Formal analysis, Project administration, Supervision, Writing - original draft, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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