



Short communication

2S albumins and nsLTP are involved in anaphylaxis to pizza sauce: IgE recognition before and after allergen processing

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ABSTRACT

Although pizza is one of the most popular foods in the world, allergic responses after ingesting pizza are relatively uncommon. However, precisely identifying the allergens responsible for these allergic reactions is challenging because of the high and diverse number of ingredients used in pizza preparation. In this report, we aim to identify the allergens responsible for systemic allergic reactions following ingestion of pizza in two patients. Using a skin prick by prick test (SPPT) and *in vitro* techniques, with natural and recombinant purified allergens from tomato and mustard seeds, we identified 2S albumin and non-specific lipid transfer proteins (nsLTP) as the proteins involved. However, IgE responses to the four nsLTPs differed before and after denaturation and reduction, thus suggesting additional complexity around nsLTP in food processing.

1. Introduction

Food allergy is a health problem rates of which have grown steadily over recent years. This rise may stem from changes in dietary habits or the use of new cooking devices and ingredients, among other factors (Cabanillas, Jappe, & Novak, 2018). Sauces used in foods such as pizza, are a complex mixture of potential food allergens, which can lead to problems in diagnosing the ingredient(s) responsible (Soller, La Vieille, & Chan, 2018). Although pizza is one of the most popular foods in the world, allergic reactions following pizza ingestion are relatively uncommon (Senna et al., 1998; Wong & King, 2004; Heffler, Guida, Badiu, Nebiolo, & Rolla, 2007) as are anaphylactic reactions associated with foods.

Precisely identifying the molecular allergens responsible for allergic reactions associated with foods is an important goal for clinical practice. However, these efforts are complicated by food processing, which can alter protein conformation, resulting in the inactivation of known immunoglobulin (Ig) E epitopes and/or the generation of new epitopes. Denaturation of proteins often exposes new or different IgE epitopes, thereby creating new risks for allergic and previously non-allergic individuals (Sathe, Teuber, & Roux, 2005; Vila et al., 2001). Conformational epitopes, which are dependent on the three-dimensional

structure of proteins, can be more susceptible to disruption by food processing (Ng, Chua, & Kuppusamy, 2014; Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015) and sequential epitopes can be altered by enzymatic hydrolysis (García-Casado, Crespo, Rodríguez, & Salcedo, 2001).

Individuals with food allergies have high IgE levels that recognize linear epitopes in allergens such as nsLTPs or 2S albumins, which are exposed after processing (Matsuo, Yokooji, & Taogoshi, 2015; Pomés, 2010). Both allergens belong to the prolamine superfamily and a shared α -helix motif maintained by inter- or intra-chain disulfide bonds (Pastorello et al., 2001). nsLTPs present hydrophobic lipid-binding cavities and are can be classified as have either nsLTP1 or nsLTP2 (Kader, 1975; Pasquato et al., 2006; Hoh, Pons, Gautier, De Lamotte, & Dumas, 2005). nsLTP1 allergy is the most frequent cause of primary food allergy and food-induced anaphylactic reactions in Mediterranean countries, and it is involved in certain cross-reactivity processes that make them important pan-allergens (Asero et al., 2000).

In this report, we present two patients who developed systemic reactions after eating pizza, aiming to identify the allergens responsible for these reactions. These individuals were identified as being sensitive to mustard and tomato, both of which are sources of important allergens, e.g. tomato seed nsLTP Sola 17 and the mustard storage protein 2S

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albumin, Sin a 1.

2. Materials & methods

2.1. Ethics statement

The study was approved by the Ethic Committee of the Hospital (PIC28/2014) and written informed consent was obtained from patient #1 and the parents of patient #2.

2.2. Patient selection and skin prick-prick test

Written informed consent was obtained from the adult (Patient #1) and the parents of the child (Patient #2). Serum samples were used for specific IgE detection assays. As a negative control, we included an atopic individual who had no past history of food allergy. This research was performed in accordance with the Ethics Guidelines of Complutense University of Madrid (Spain).

Two patients who developed symptoms immediately after eating a slice of pizza were included in the study. Patient #1 was a 21 year-old man referred for assessment after two episodes of systemic reactions, including intense erythema, generalized urticaria, and angioedema of the hands within minutes of eating a slice of pizza. Skin prick prick test (SPPT) with the components of pizza was positive for tomato sauce (7×5 mm), but negative for cheese, tuna and pizza dough. The patient has a positive SPPT test to the peel (30×11 mm) and pulp (6×5 mm) of raw (non-processed) tomato. Patient #2 was a 3.5 year-old child with a history of walnut allergy. He experienced oral allergy symptoms, abdominal pain, and urticaria after eating chicken pizza with barbecue sauce. The child tolerated chicken ingestion at a later date. SPPT for pizza components was positive for barbecue sauce, but negative for raw tomato peel and pulp. Skin prick test (SPT) was positive (7×4 mm) for mustard (BIAL, Zamudio; Spain) but negative for egg, prawn, chicken, wheat, hazelnut, chestnut, soybean, peanut (Laboratorios LETI, S.L.U.; Tres Cantos, Spain), milk, peach, and *Anisakis simplex* (ALK-Abelló, Madrid, Spain).

2.2.1. Protein extracts and recombinant and natural proteins

In vitro assays were performed with extracts from tomato peel, pulp, and seeds, as well as yellow (*Sinapis alba*) and oriental (*Brassica juncea*) mustard-seed extracts prepared manually in our laboratory, as previously described (Martín-Pedraza et al., 2016; Palomares et al., 2005; Wal, 2003).

A panel of natural (*n*) and recombinant (*r*) allergens was also used. Natural allergens were obtained by saline extraction from tomato peel or yellow mustard seeds. They were purified through one Sephadex G-50-column using gel filtration chromatography as well as a RP-HPLC (Shimadzu, Kyoto, Japan) by means of a C-18 reverse phase column (Hichrom, Theale, UK), with an acetonitrile gradient (ACN) from 0% to 60%, at 1 ml/min.

We produced the recombinant allergens-rSola l 7 (tomato seed nsLTP1, 10 kDa), rSola l 6 (tomato seed nsLTP2, 7 kDa) and rSin a 3 (mustard seed nsLTP1, 10 kDa) – from *Pichia pastoris* extracellular medium after 72 h of methanol induction. These were then purified by anion exchange chromatography (DEAE-cellulose), -gel filtration chromatography (Sephadex G-50), and RP-HPLC, as described by Martín-Pedraza et al. (2016).

nSin a 1 allergen (2S albumin, 15 kDa) was purified from mustard seeds as described by Vereda et al. (2011), and the natural allergen nSola l 3 (nsLTP1, 10 kDa) was obtained from tomato peel and pulp following the procedure described by López-Matas et al. (2015). Unless indicated otherwise, ACN and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. ELISA and immunoblotting IgE assays

Polystyrene 96-wells (Costar, Fisher scientific, Madrid, Spain) were coated with 2 µg/ml of each purified protein. The procedure previously described by Vereda et al. (2011) was performed with a 1:5 serum dilution and revealed with the system based in o-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, St. Louis, MO, USA) in 0.05 M phosphate-citrate buffer. The reaction was stopped with the addition of 4 M HCl. Absorbance was measured at 492 nm using an iMark Microplate Absorbance Reader (Bio-Rad, Irvine, CA, USA) and non-specific binding of antibodies was eliminated by subtracting the absorbance for control wells from the absorbance for antigen-coated wells. Measurements were made in triplicate for three independent assays.

Immunoblots were carried out with 20 µg of each extract or 1 µg of purified (allergenic) protein on 17% SDS-PAGE gels in the presence or absence of β-mercaptoethanol (βME) as a reducing agent. The extracts were denatured for 20 min at 90 °C and transferred onto nitrocellulose membranes. Assays were performed using serum (diluted 1:5) from both patients, as previously described (Palomares et al., 2006). The signal was developed with the chemiluminescent ECL-Western blotting reagent (Amersham Biosciences, Barcelona, Spain) and assayed using an LAS-3000 luminescent image analyzer (General Electric Healthcare, Madrid, Spain).

For immunoblotting inhibition experiments, 20 µg each of tomato peel and yellow mustard seed extracts were incubated with serum from patient #2 (diluted 1:5) previously mixed with 2 µg of purified nsLTPs (Sola l 3 or Sin a 3) or BSA as control. Otherwise, the protocol was the same as described above. The signal was assayed using ECL Western blotting reagent (Amersham Biosciences, Barcelona, Spain). Volumnograms of the reactive bands were analyzed in duplicate from two independent experiments using scanning densitometry (UVI Band V97, UVitec. St. John's Innovation Center, Cambridge, UK).

3. Results and discussion

3.1. IgEs recognize pizza-sauce allergens

The clinical cases presented here concern episodes of systemic reactions after eating a slice of pizza. Neither individuals reacted to most of the pizza components analyzed, with the exception of the tomato sauce. The serum of patient #1 strongly recognized IgE-binding bands in tomato peel and pulp extracts after immunoblotting as well as in both yellow (*Sinapis alba*) and oriental (*Brassica juncea*) mustard extracts. A double IgE immunoreactive band of 10 kDa was detected in tomato peel and pulp extracts, and three bands around 30, 15, and 10 kDa were visible in both mustard-seed extracts (Fig. 1A). No IgE-reactive signal was observed in the tomato-seed extract. Regarding patient #2, only bands of 15 and 30 kDa were detected in both mustard extracts (Fig. 1B). A band of around 40 kDa was visible in tomato extracts and in yellow mustard extract. However, the same molecular mass band was visible when non-atopic serum was used as a control and the signal was considered anon-specific (Data not shown).

When Sin a 1, a 2S albumin, and Sin a 3, Sola l 3, Sola l 7, and Sola l 6 were tested in immunoblotting or ELISA assays, specific IgEs from the serum of patient #1 recognized Sin a 1, Sin a 3, and Sola l 3. Neither Sola l 7 nor Sola l 6 were detected. Patient #2 only recognized the double band of 15 and 30 kDa of purified Sin a 1 corresponding to the monomer and dimer, respectively. These results indicate that patient #1 is allergic to barbecue sauce due to mustard and tomato pulp/peel while the symptoms presented by patient #2 allergy to the mustard spice included in the sauce (Fig. 2). These results are consistent with those obtained in ELISA tests using the same proteins with the following values of A_{492nm} : Sin a 1 (0.55), Sin a 3 (0.95), and Sola l 3 (0.21) for patient #1 and Sin a 1 (0.18) for patient #2. Values for absorbance measurements at 492 nm were positive for results greater than three-fold the standard deviations above the mean of controls from non-

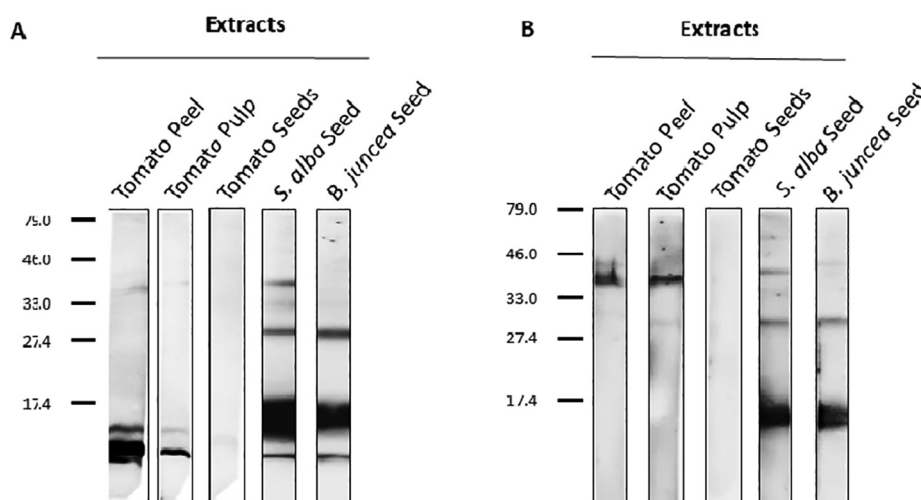


Fig. 1. Immunoblot with extracts, 20 μ g of tomato peel, tomato pulp, tomato seeds, and mustard seeds (*S. alba* and *B. juncea*) incubated with serum patient #1 (A) and serum patient #2 (B) 1:5 diluted.

atopic donors (in these assays above 0.1). Nevertheless, when purified nsLTPs were subjected to immunoblotting under reducing conditions, IgE recognition of Sola l 7, Sola l 3, and Sin a 3 with both sera were observed. IgE recognition of epitopes that are uncovered after protein denaturation either through the breakdown of disulphide bonds as a result of food processing or by exposure to the digestive tract environment during ingestion is a frequent event in food allergy (Pastorello et al., 2003). Thus, the recognition of these internal regions within the different nsLTPs, which are exposed when they are completely denatured could explain reactions to sauces prepared under thermal treatments such as ketchup or barbecue sauce. To summarize, the recognition of Sin a 1 is common in both patients, whereas LTPs from mustard, Sin a 3 and Sola l 3 from tomato was only recognized by patient #1. This is why this patient's SPPT was positive for natural tomato whereas patient #2 showed a negative reaction. Clinicians may be misled when diagnosing patients due to the differences between diagnostic tools used in routine clinical practice (e.g. SPT, SPPT, *in vitro* studies by immunoCAP) in which proteins are used in native conditions, and other tools such as immunoblotting, where proteins are frequently denatured, thus producing a different IgE recognition of allergens (Brenna et al., 2000; Lindorff-Larsen & Winther, 2001; Koppelman, Hefle, Taylor, & De Jong, 2010).

3.2. Detection of the potential primary nsLTP sensitizer

In order to elucidate which nsLTP allergen could be the primary sensitizer in patient #1, inhibition experiments were carried out using tomato and mustard extracts transferred to membranes and Sola l 3 and Sin a 3 as inhibitors. IgE binding to a 10-kDa molecular mass band of mustard-seed extract was completely blocked with Sin a 3, compared to

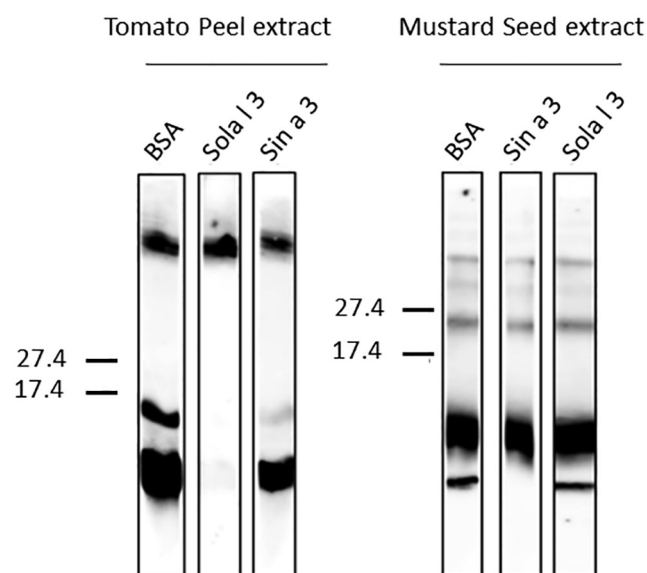


Fig. 3. Inhibition experiments with 20 μ g of tomato peel and mustard seed extracts, and 2 μ g of purified Sola l 3 and Sin a 3 as inhibitors were performed. The inhibition percentage is shown in the bottom of the figure.

the barely visible inhibition when Sola l 3 was used as inhibitor. However, while Sola l 3 was able to completely abolish IgE binding to 10 and 15 kDa proteins from tomato peel extract, Sin a 3 elicited a 55% inhibition (Fig. 3).

Therefore, both patients were allergic to pizza sauce. Sin a 1 and

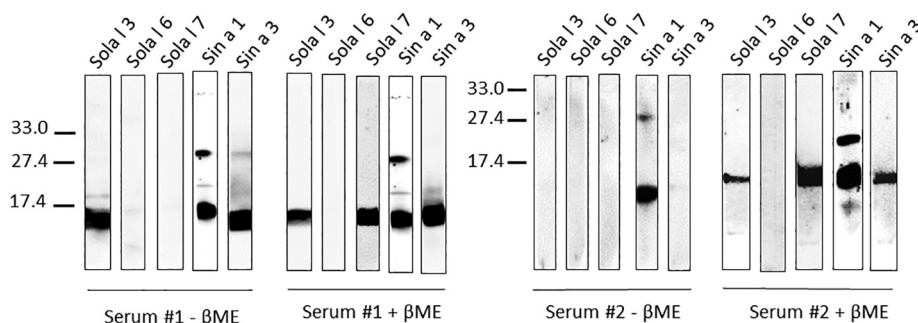


Fig. 2. Immunoblots incubated with purified allergens (1 μ g) with serum patient #1 and serum patient #2 with and without reducing agent (β ME).

nsLTP1 (Sin a 3 and Sola l 3) act as sensitizers for patient #1, whereas Sola l 7 seems to share linear IgE epitopes with the other nsLTPs. Sin a 1 was apparently the only allergen responsible for sensitization of patient #2, however once again new IgE epitopes are uncovered when a denaturing agent is used in immunoblotting. These results suggest that both mustard and tomato nsLTPs contributed to the sensitization in patient #1 with partial IgE cross-reactivity between them. However, the possible sensitization of this patient to tomato and mustard nsLTPs by cross-reactivity to other nsLTP should not be ruled out. These positive results agree with those from Asero et al. (2002) and Mothes-Luksch et al. (2017), where allergic reactions to plant-derived food in Southern Europe were found to be associated with systemic reactions and cross-reactivity with onion, eggplant, mustard, tomato, or pepper.

In the Mediterranean area, nsLTPs have been described as major clinically relevant allergens in numerous plant-derived foods (Hartz et al., 2010). In Spain, nsLTPs from peach and cherry induce positive SPT in more than 89% of patients sensitized to each of these fruits (Ballmer-Weber, 2002). Moreover, members of the allergenic nsLTP family have also been identified in other plant foods such as peanut, mustard, tomato, lentil, and maize (Lauer et al., 2009; San Miguel-Moncin et al., 2003; Sirvent, Palomares, Cuesta-Herranz, Villalba, & Rodríguez, 2012; Pastorello et al., 2004). Recent studies on nsLTP cross-reactivity have shown that most patients experience adverse reactions after ingestion of other non-botanically related plant foods that have low sequence similarity percentages. This fact could be determined by the great similarity in tertiary structures between nsLTPs from different plant sources, or because of low affinity interactions in certain IgE epitopes. However, in terms of why patients tolerate some foods or develop severe adverse reactions with other nsLTPs, the presence of linear or conformational epitopes, could be an explanation (Morales, López-Matas, Moya, & Carnés, 2014). Nevertheless, from a clinical perspective, cross-reactivity through these panallergens involves highly problematic sensitivities with numerous plant species.

In summary, we report two cases of severe pizza allergy in which the sauce was the allergenic inducer. These cases further reinforce the need to carefully investigate the composition of multi-ingredient dishes, in order to identify unusual or hidden allergens or epitopes. Moreover, barbecue sauce on a pizza can contain several components, in this case mustard and tomato, and allergic patients with similar symptoms to this food may be sensitized against different allergens from these components by co-sensitization or cross-reactivity with other allergens. This finding underscores the need for the most complete panel of purified allergens possible to allow for accurate diagnosis. In addition, thorough knowledge of the structural characteristics of these protein allergens is necessary for correct manipulation. We highlight SPPT as a useful tool to identify IgE against allergens from specific tissues; the fact that these allergens are poorly represented in the fruit may make it difficult to detect them with commercial allergenic extracts.

Another key point emerging from this study is the implication of different nsLTPs belonging to the same fruit in these allergies, evidencing the already reported lack of cross-reactivity between them (Vereda et al., 2011). In addition, Sin a 3 was also involved in sensitizing patient #1 exhibiting partial cross-reactivity to Sola l 3 and manifesting once again the complexity of nsLTP and finally the importance of using techniques such as immunoblotting or ELISA appropriately, for instance in the absence or presence of denaturing and reducing conditions. Immunoblotting is a non-quantitative assay, and as such does not allow absolute evaluation of the results, as these need to be complemented with ELISA techniques. For good interpretation of allergenicity results in food products subjected to industrial processing, it is necessary to use a reducing agent, simulating the thermal processing to which that food, and therefore, the allergens contained in that source, is exposed.

4. Conclusion

The presence of certain ingredients in processed foods that may

contain potential allergens, reinforces the need for the most complete panel of purified proteins as possible to enable a more accurate diagnosis. The pizza sauce ingredients analysed in this study can lead to severe and unexpected reactions caused by allergens belonging to the 2S albumins or nsLTPs protein families. Both families include some of the most relevant allergens both in terms of severity and impact on the quality of life of patients. On the other hand, the complexity of the nsLTP family, which has been proven in these clinical cases, required correct use of *in vitro* diagnostic techniques in both native and reducing conditions, in order to use these purified allergens in a way that most closely resembles real food processing.

CRedit authorship contribution statement

Laura Martín-Pedraza: Conceptualization, Methodology, Investigation, Writing - original draft, Visualization, Writing - review & editing. **Andrea Wangorsch:** Supervision, Resources. **Cristina Bueno-Díaz:** Investigation. **Manuel de las Heras:** Resources. **Stephan Scheurer:** Supervision, Resources. **Javier Cuesta-Herranz:** Supervision, Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing. **Mayte Villalba:** Supervision, Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing.

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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Ethical approval reference n° was included and written informed consent for this study was obtained from patients.

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