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Detection of a novel 20 kDa shrimp allergen showing cross-reactivity to house dust mites

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KEY WORDS

Tropomyosin, 20 kDa allergen, crustacean shellfish allergy, immunoblotting, cross-reactivity

SUMMARY

Background: Allergy to crustacean shellfish is one of the most common IgE-mediated food allergies, and tropomyosin has been identified as the major allergen. However, not all subjects affected by this allergy are IgE-positive to tropomyosin. **Aims:** To evaluate whether sera of patients with shrimp allergy but negative for tropomyosin react to other allergen(s); and to evaluate the role such allergen(s) may play in cross-reactivity between crustaceans and house dust mites (HDMs). **Methods:** Three different pools of sera—one from subjects with shellfish allergy and HDMs positivity, but negative for recombinant and native tropomyosin (rPen a 1 and nPen m 1) (Pool 2); a second from subjects with tropomyosin and HDMs positivity (Pool 1); and the last from subjects allergic only to HDMs (Pool 3) were submitted to immunoblotting. Subsequently, a 20 kDa protein-enriched fraction of shrimp extract was used at two different concentrations (10 and 100 µg/mL) to pre-absorb the Pool 2 serum and to evaluate, by ELISA assay, the level of inhibition on shrimp and HDMs-coated wells, respectively. **Results:** The Pool 2 serum showed IgE reactivity against a 20 kDa component. Its pre-absorption with an enriched fraction of 20 kDa protein caused an inhibition of 56% in IgE binding to shrimp extract at a concentration of 100 µg/mL, and of 14% and 35% to HDMs extract at concentrations of 10 and 100 µg/mL, respectively, as measured by ELISA assay. **Conclusions:** The 20 kDa component seems to be a new crustacean allergen and it could play a role in cross-reactivity with HDMs.

Abbreviations:

HDM, house dust mites; ELISA, enzyme-linked immunosorbent assay; SPT, skin prick tests

Introduction

Allergy to crustacean shellfish (shrimp, crab, lobster) is one of the most common IgE-mediated food allergies and is often associated with severe reactions. Tropomyosin, a highly conserved and heat-stable myofibrillar protein of

35–38 kDa has been identified as the major allergen from crustaceans (1). Moreover, many studies have suggested that tropomyosin is also present in house dust mites (HDMs) (2), cockroach (3), squid (4), and other molluscs (5) and it may be responsible for cross-reactivity among different shellfish, between cockroach and HDMs, and between crustaceans and HDMs. For this reason, the tropomyosin molecule can be considered a pan-allergen of invertebrates (6).

In the last few years, *in vitro* assays for detection of specific IgE against recombinant tropomyosin from *Penaeus*

aztecus (rPen a 1) or against native purified tropomyosin from *Penaeus monodon* (nPen m 1) have been developed, and are increasingly used for molecular diagnosis of shellfish allergy in clinical practice. However, in our experience, about 20% of subjects with HDMs sensitisation as confirmed by *in vitro* and *in vivo* assays and showing allergic symptoms after crustacean ingestion resulted IgE negative to rPen a 1 or nPen m 1. This observation suggests that other components might play a role in the cross-reactivity between crustaceans and HDMs. The aim of our study was, therefore, to evaluate whether the sera of these patients were able to recognize allergen(s) other than tropomyosin, and whether such allergen(s) play(s) a role as a cross-reactive allergen between crustaceans and HDMs.

Material and methods

Sera of 21 patients with both SPTs (ALK Abellò, Madrid, Spain) and IgE (Phadia, ImmunoCAP, Uppsala, Sweden) positivity for HDMs and shrimp extract were also tested for IgE against recombinant (rPen a 1, Phadia, ImmunoCAP) and natural (nPen m 1, DPC, Immulite 2000, Siemens, Erlangen, Germany) tropomyosins. Five of the sera scored negative for both these types of tropomyosins; they were pooled (Pool 2) and tested by immunoblotting (IB) in comparison with two other pools: a pool of five sera selected from the tropomyosin IgE-positive patients (Pool 1), and a pool of five sera from patients who were IgE-positive only for HDMs (Pool 3). Of the five patients from Pool 2 with crustacean allergy but negative for tropomyosins, two presented an oral allergic syndrome (OAS) as the clinical manifestation, while one presented both OAS and rhinitis and two presented urticaria-angioedema. Unlike patients with positivity for tropomyosins (Pool 1), none presented asthma or anaphylaxis. The limited number of subjects, however, does not allow for defining significant differences in the clinical presentation of the two populations.

Preparation of crude shrimp extract

Peeled shrimps were homogenized and submitted to an aqueous extraction in 0.1M phosphate-buffered saline, pH 7.4 (PBS) by shaking for 16 hours at 4 °C. The suspension was centrifuged at 3000 g for 30 minutes at 4°C and corresponding supernatant was filtered through a 0.45- μ m membrane. Protein content was 3.2 mg/ml as

measured according to Bradford (7) by the Bio Rad method (BioRad, Milan, Italy).

Purification of tropomyosin and of a 20 kDa component from shrimp

Peeled shrimps were snap-frozen and ground in a mortar. 5g of the resulting powder were added to 50 ml of extraction buffer (1 M KCl and 0.5 mM DTT, pH 7.0). The mixture was left for 16 hours at room temperature. After centrifugation at 5000g for 15 minutes, the supernatant was cooled to 4°C and its pH adjusted to 4.6 with HCl 1M, leaving the sample under stirring for 30 minutes until a precipitate (representing the tropomyosin-enriched fraction) was obtained. The precipitate was then dissolved in extraction buffer, and both the precipitate and the supernatant (representing the 20 kDa-enriched component) were dialyzed against PBS before use.

Immunoblotting (IB) and IB-inhibition

The three different pool samples were first checked on shrimp extract by IB under reducing conditions according to Towbin (8). IB and IB inhibition experiments were performed as previously described (9). Briefly, shrimp extract was mixed with LDS sample buffer (Nupage Bis-Tris, Novex, Prodotti Gianni, Milan, Italy) and 5% β -mercaptoethanol. The sample was heated at 100°C for 5 minutes before being submitted to electrophoresis run (25 μ g/lane) in a 10% polyacrilamide precast gel (Nupage Bis-Tris) at 180 mA for 1 hour. The resolved proteins were transferred onto a nitrocellulose membrane and left to rest for 1 hour. The membrane was then saturated with 0.1 mol/L Tris-buffered saline containing 5% fat-free milk powder and incubated for 16 hours at 4°C with 700 μ l of the serum pool and 500 μ l of saturation buffer. After three consecutive washings, bound specific IgE were detected by peroxidase-conjugated anti-human IgE antibodies goat serum (Biospecific, Emeryville, CA) diluted to 1:3500 in saturation buffer, using an ECL western blotting kit (Amersham, Milan, Italy). In inhibition studies, pool 1 was pre-absorbed with 100 μ g of an enriched fraction of tropomyosin obtained as previously described.

ELISA inhibition assay

ELISA inhibition assays were performed as previously described (10). For the coating phase, two micrograms/100 μ l (coating buffer: 15 mmol/L Na₂CO₃ and 35 mmol/L

NaHCO₃, pH 9.6) of mite extract or 2 µg/100 µL of shrimp extract were used per well of 96-microtitre plates (Maxisorp Nunc, Roskilde, Denmark). After washings, wells were saturated with 2% bovine serum albumin (BSA) in PBS for 2 hours at room temperature, and then washed again before being dried until use. In parallel, for pre-absorption experiments, 100 µL of Pool 2 were added to tubes containing one of the following: for inhibition of IgE response to shrimp extract, 100 µL of 20 kDa enriched fraction (100 µg/mL), as inhibitor, or 100 µL of PBS, as control; and for inhibition of IgE response to HDMs, 100 µL of 20 kDa-enriched fraction, at two different concentrations (10 and 100 µg/ml); 5 µg of HDMs extract, 5 µg of an unrelated extract (Grass), as inhibitors, or 100 µL of PBS, as control. Pre-absorption was prolonged for 2 hours at room temperature. A 100-µL of sample from each tube was collected and added to the corresponding well and incubated for 2 more hours. After washings, specific IgE was detected by a peroxidase-conjugated anti-human IgE from goat (diluted 1:1500) (Biospacific) and the absorbance values were read spectrophotometrically at 450 nm. The percentage of inhibition was calculated on the basis of the absorbance value of the corresponding control.

Results

Immunoblot analysis of Pool 1 serum showed strong reactivity against components of the shrimp extract, ranging between 30 and 43 kDa (Fig. 1, line 1). In particular, a component of about 38 kDa, corresponding to tropomyosin, was recognized as shown by the almost complete disappearance of such reactivity when the serum pool was pre-incubated with 100 µg of tropomyosin-enriched fraction (Fig. 2, lane 2). In contrast, subjects with positivity for HDMs and shrimp extract, but negative for rPen a 1 and nPen m 1 (pool 2), showed IgE reactivity mainly against the 20 kDa component (Fig. 1, lane 3). The preincubation of this serum pool with the 20 kDa protein-enriched fraction, at a concentration of 100 µg/mL, caused an inhibition of 56% of IgE binding to shrimp extract, as shown by ELISA inhibition tests (Fig. 3, column 1). Even if the inhibition of IgE binding resulted incomplete, probably because of an insufficient amount of inhibitor, our experiments indicate that the 20 kDa component could be a new shrimp allergen. In addition, IgE binding to HDM extract of the same pool after preincubation with two different concentrations of en-

Figure 1 - Lane 1, SDS-PAGE of shrimp extract; Lane 2, IgE-reactivity of pool 1; Lane 3, IgE-reactivity of pool 2; Lane 4, IgE-reactivity of pool 3; Lane 5, IgE-reactivity of non atopic serum

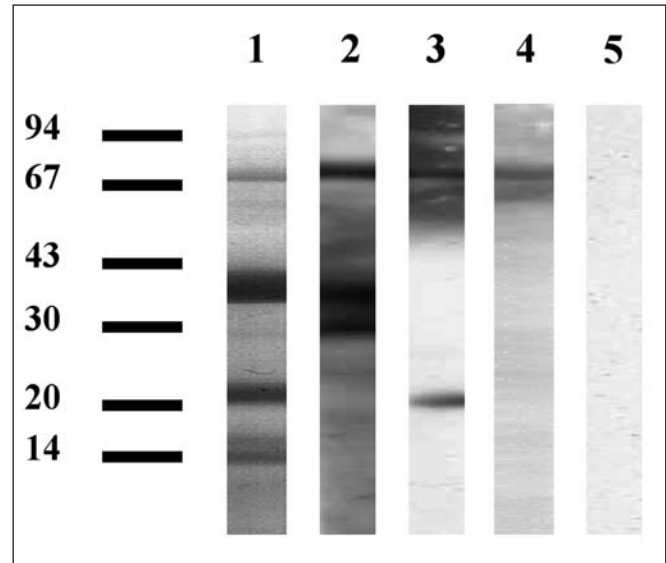


Figure 2 - Lane 1, IgE-reactivity of pool 1 on shrimp extract; Lane 2, inhibition with 100 µg of a fraction enriched of tropomyosin

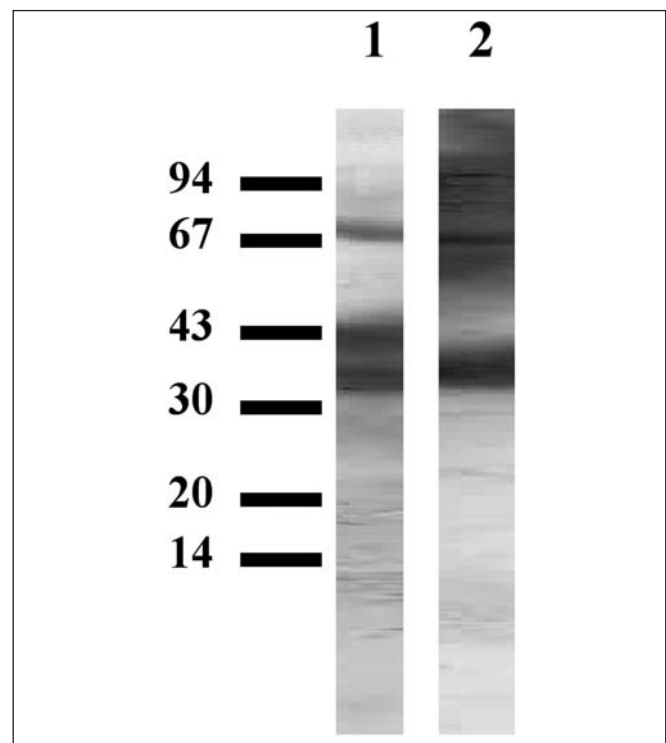
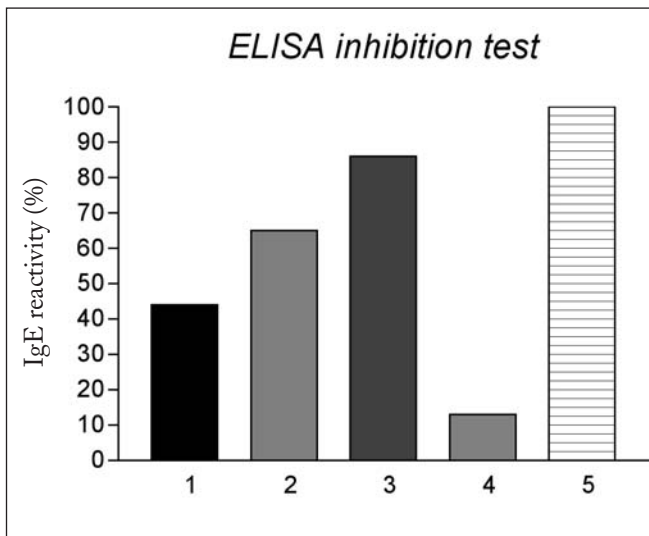


Figure 3 – IgE reactivity to shrimp extract (1) after pre-absorption of pool 2 with a 20 kDa enriched fraction (100 µg/mL). IgE reactivity to HDMs extract after pre-absorption of pool 2 with a 20 kDa enriched fraction at (2) 100 µg/mL and at (3) 10 µg/mL concentrations; (4) with 5 µg of HDMs extract and (5) with 5 µg of an unrelated Grass extract



riched fraction of 20 kDa protein (10 µg/mL and 100 µg/mL) was partially inhibited in a dose-dependent manner (14% and 35%, respectively), suggesting that this allergen is also present in HDM extract.

Discussion

In this study we demonstrated that cross-reactivity between HDMs and crustaceans might also be due to the presence of a 20 kDa component of shrimp extract. Such a component seems involved as a cross-reacting molecule only in a subset of patients with crustacean allergy. The results are very similar to those reported in the recent paper of Shiomi *et al.* (11). In their study, 8 out of 16 sera from crustacean-allergic patients showed reactivity against a 20 kDa allergen, identified as a sarcoplasmic calcium-binding protein (SCP), and probably limited to shrimp and crayfish. More recently, Ayuso *et al.* (12), on 21 out of 38 sera of patients with immediate allergic reaction to shrimp, showed an IgE binding to a 20 kDa shrimp component that they identified as a myosin light chain (MLC) called Lit v 3.0101. They also demonstrated that the amino acid sequence of MLC is 66% similar to cockroach MLC of *Blattella germanica* (Bla g 8). On the basis

of the molecular weight deduced by our IB experiments, we could speculate that SCP, Lit v 3.0101 and our 20 kDa component might be the same molecule. More studies on 20 kDa component at the level of amino acid sequence must to be performed to confirm this possibility.

Moreover, we observed that pre-incubation of Pool 2 with an enriched fraction of 20 kDa component inhibited the IgE binding to both shrimp and HDM extracts, although inhibition was less for HDM than for shrimp (35% vs 56%, fig.3). Our observations confirm, however, the presence of the 20 kDa protein in HDMs, and might explain how all patients allergic to crustaceans and positive for 20 kDa protein – similar to findings reported in the study by Ayuso *et al.* – also present sensitization to HDMs.

In conclusion, we identified a new allergen correlated with crustacean allergy and HDM cross-reactivity. Since some patients are positive only for this allergen, it is important to add it to the component-resolved diagnosis methods for shellfish allergy to avoid the loss of some positivities.

References

- Shanti KN, Martin BM, Nagpal S, Metcalfe DD, Sabba-Rao PV. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE binding epitopes. *J Immunol* 1993; 151: 5354-63.
- Aki T, Kodama T, Fujikawa A, Miura K, Shigeta S, Wada T, et al. Immunochemical characterization of recombinant and native tropomyosin as a new allergen from house dust mite, *Dermatophagoides farinae*. *J Allergy Clin Immunol* 1995; 96: 74-83.
- Asturias JA, Arilla MC, Gomez-Bayon N, Martinez A, Palacios R, Martinez J. Molecular characterization of American cockroach tropomyosin (Per a 7), a cross-reactive allergen. *J Immunol* 1999; 162: 4342-8.
- Miyzawa H, Fukamachi H, Inagaki Y, et al. Identification of the first major allergen of a squid (*Todarodes pacificus*). *J Allergy Clin Immunol* 1996; 98: 948-53.
- Leung PSC, Chow WK, Duffney S, Kwan HS, Gershwin E, Chu KH. IgE reactivity against a cross-reactive allergen in crustacean and mollusca. Evidence for tropomyosin as the common allergen. *J Allergy Clin Immunol* 1996; 98: 954-61.
- Reese G, Ayuso R, Lehrer SB. Tropomyosin: an invertebrate pan-allergen. *Int Arch Allergy Immunol* 1999; 119: 247-58.
- Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-54.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc Natl Acad Sci* 1979; 76: 4350-4.
- Borghesan F, Mistrello G, Roncarolo D, Amato S, Plebani M, Asero R. Respiratory allergy to lipid transfer protein. *Int Arch Allergy Immunol* 2008; 147: 161-5.

10. Asero R, Amato S, Alfieri B, Folloni S, Mistrello G. Rice: another potential cause of food allergy in patients sensitized to lipid transfer protein. *Int Arch Allergy Immunol* 2007; 143: 69-74.
11. Shiomi K, Sato Y, Hamamoto S, Mita H, Shimakura K. Sarcoplasmic calcium-binding protein: identification as a new allergen of the black tiger shrimp *Penaeus monodon*. *Int Arch Allergy Immunol* 2008; 146: 91-8.
12. Ayuso R, Grishina G, Bardina L, Carillo T, Blanco C, Ibanez MD, et al. Myosin light chain is a novel shrimp allergen, Lit v 3. *J Allergy Clin Immunol* 2008; 122: 795-802.