M.G. Giuffrida[1], D. Villalta[2], G. Mistrello[3], S. Amato[3], R. Asero[4]

Shrimp allergy beyond Tropomyosin in Italy: clinical relevance of Arginine Kinase, Sarcoplasmic calcium binding protein and Hemocyanin

[1]Istituto Scienze Produzioni Alimentari CNR, c/o BioIndustry Park S. Fumero, Colleretto Giacosa, (TO), Italy
[2]Allergologia e Immunologia Clinica, Dipartimento di Medicina di Laboratorio, A.O. "S. Maria degli Angeli", Pordenone, Italy
[3]Lofarma SpA, R & D, Milano, Italy
[4]Ambulatorio di Allergologia, Clinica San Carlo, Paderno Dugnano (MI), Italy

Key words

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Corresponding author
Riccardo Asero
Ambulatorio di Allergologia
Clinica San Carlo
Via Ospedale 21
20037 Paderno Dugnano (MI), Italy
Phone: +39 02 990 38 470
Fax: +39 02 990 38 223
E-mail: r.asero@libero.it

Summary

Background. *Little is known about the prevalence and clinical relevance of sensitization to shrimp allergens other than tropomyosin.* ***Objective.*** *We detected the prevalence of arginine kinase and sarcoplasmic calcium binding protein sensitization, and identified a high molecular weight allergen that is frequently recognized by Italian shrimp-allergic patients.* ***Methods.*** *Sera from 40 shrimp-allergic patients underwent the detection of IgE specific for arginine kinase (rPen m 2) and sarcoplasmic calcium-binding protein (rPen m 4) by ISAC 112 Microarray platform and immunoblot analysis. A high molecular weight shrimp allergen was identified by N-terminal amino acid sequencing.* ***Results.*** *IgE to rPen m 2 and rPen m 4 were found in 4/40 (10%) and 6/40 (15%) sera, respectively; two sera reacted to both allergens. Clinically, 6/8 Pen m 2 and/or Pen m 4 reactors experienced severe allergies to shrimp. On immunoblot, 4/6 rPen m 4-positive sera showed IgE reactivity at about 20 kDa, whereas no rPen m 2-positive serum reacted at about 40 kDa. Nineteen (47%) sera showed IgE reactivity at molecular weights > 60 kDa. Such profile was not associated with IgE reactivity to rPen m 2 or rPen m 4. N-terminal amino acid sequencing of the high molecular weight allergen led to the identification of hemocyanin.* ***Conclusion.*** *Shrimp arginine kinase and sarcoplasmic calcium-binding protein are minor allergens sensitizing only 10% -15% of Italian shrimp-allergic patients, but are clinically relevant. Hemocyanin is a clinically relevant high molecular weight shrimp allergen possibly cross-reacting to house dust mite.*

Introduction

Shrimp is a frequent cause of food allergy at all latitudes. Besides tropomyosin, a major allergen that was identified as long as 18 years ago (1-3), several other allergenic proteins have been detected in recent years, including arginine kinase (4,5), sarcoplasmic calcium binding protein (6,7), and myosin light chain (8). Very recently, hemocyanin was identified as one further allergen in a freshwater shrimp as well in other crustaceans (9,10) along with troponin C (10,11), triosephosphate isomerase (11), and fatty acid binding protein (FABP) (10). Little is known about the prevalence of sensitization to these new allergens as well as about their clinical relevance. In a recent multi-centre study on more than 100 Italian shrimp-allergic adult patients investigated by immunoblot analysis and rPen a 1-specific IgE measurements, only 41% were tropomyosin reactors, whereas IgE reactivity at molecular weights > 60 kDa was detected in 52% of cases (12). In contrast, IgE reactivity at the molecular weights of arginine kinase (Pen m 2; 40 kDa), sarcoplasmic calcium binding protein (Lit v 4; 20 kDa), myosin light chain (Lit v 3; 20 kDa), triosephosphate isomerase (Cra c 8; 27 kDa), troponin C (Cra c 6; 17 kDa), and fatty acid binding protein

		___SPT___		__CAP___		____ISAC_____		
No.	**History**	**Shrimp**	**Mite**	**Shrimp**	**Pen a 1**	**Pen m 1**	**Pen m 2**	**Pen m 4**
1	d	Pos	Pos	12,8	3,92	10	Neg	Neg
2	ab	Pos	Pos	1,71	0,63	4,3	Neg	Neg
3	X	Pos	Pos	9,95	Neg	Neg	4	4,2
4	ab	Pos	Pos	ND	0,24	0,7	Neg	Neg
5	b	Pos	Pos	ND	Neg	neg	0,8	neg
6	b	Pos	Pos	ND	0,28	0,6	Neg	Neg
7	b	Pos	Pos	ND	Neg	Neg	Neg	2,3
8	b	Pos	Pos	ND	Neg	Neg	Neg	0,8
9	ab	Pos	Pos	ND	Neg	Neg	Neg	Neg
10	b	Pos	Pos	ND	Neg	Neg	Neg	Neg
11	ab	Pos	Neg	ND	Neg	Neg	Neg	1
12	b	Pos	Pos	ND	Neg	0,4	Neg	Neg
13	a	Pos	ND	ND	Neg	Neg	Neg	Neg
14	a	Pos	Pos	1,69	Neg	Neg	2,5	Neg
15	b	Pos	ND	13,2	0,75	2,6	Neg	Neg
16	a	Pos	Pos	14,8	0,55	3	Neg	Neg
17	x	Pos	Pos	ND	Neg	Neg	Neg	Neg
18	x	Pos	Pos	ND	Neg	Neg	Neg	Neg
19	x	Pos	Pos	ND	Neg	Neg	Neg	Neg
20	b	Pos	Pos	ND	56,2	77	Neg	Neg
21	a	Pos	Neg	ND	50,6	86	Neg	Neg
22	a	Pos	Neg	ND	Neg	Neg	Neg	Neg
23	b	Pos	Neg	ND	Neg	Neg	Neg	Neg
24	b	Pos	Pos	3,12	Neg	Neg	1,1	1,3
25	b	Pos	Pos	7,88	Neg	Neg	Neg	Neg
26	b	Pos	Neg	ND	Neg	Neg	Neg	Neg
27	b	Pos	Pos	ND	Neg	Neg	Neg	Neg
28	b	Pos	Pos	0,18	Neg	Neg	Neg	Neg
29	a	Pos	Pos	ND	Neg	Neg	Neg	Neg
30	a	Pos	Pos	ND	Neg	Neg	Neg	Neg
31	bd	Pos	Pos	ND	Neg	Neg	Neg	Neg
32	a	Pos	Pos	0,71	Neg	neg	Neg	Neg
33	a	Pos	Pos	ND	Neg	Neg	Neg	Neg
34	b	Pos	Pos	ND	Neg	Neg	Neg	Neg
35	a	Pos	ND	20,0	0,14	Neg	Neg	0,6
36	bd	Pos	Pos	ND	Neg	Neg	Neg	Neg
37	bd	Pos	Pos	ND	Neg	Neg	Neg	Neg
38	a	Pos	Neg	ND	Neg	Neg	Neg	Neg
39	a	Pos	Neg	ND	Neg	Neg	Neg	Neg
40	b	Pos	Neg	ND	Neg	Neg	Neg	Neg

History: a = oral allergy syndrome; b = urticaria/angioedema; d = rhinitis and/or asthma; x = anaphylaxis. Pen a 1: IgE to tropomyosin detected by ImmunoCAP. Pen m 1, Pen m 2, and Pen m 3: IgE to different shrimp allergens by ISAC 112 microarray.

Table 1 *- Detection of IgE reactivity to Pen a 1, Pen m 1, Pen m 2 and Pen m 4 in 40 shrimp-allergic Italian patients.*

(15 kDa) was rather rarely observed (13% altogether). Due to the absence of a routine assay able to detect IgE reactivity to the new minor allergens the analysis was not pursued further. Now, recombinant arginine kinase and sarcoplasmic calcium binding protein are available as a routine diagnostic means in ISAC microarray assay (Thermofisher, Phadia, Uppsala, Sweden). Thus, we measured IgE specific for these two allergens in sera from a group of shrimp-allergic patients included in the previous study, in order to assess their prevalence and clinical relevance and to check whether the high molecular weight allergens detected in the previous study were polymers of Pen m 2 or Pen m 4. Further, since high molecular weight shrimp allergens are not currently available for in-vitro shrimp allergy diagnosis, the N-terminal sequencing of the high molecular weight allergen was carried out as well.

Patients and Methods

Patients

The clinical features of patients have been described in detail before (12). Briefly, the starting population consisted of 116 adults who were selected in 15 Italian allergy centres from June to December, 2009 based on unequivocal clinical history of shrimp allergy confirmed by positive skin prick tests (SPT) with fresh material and/or commercial shrimp extract. In most cases, patients experienced systemic symptoms (urticaria with or without angioedema, asthma, or anaphylaxis) following the ingestion of shrimps. Since many of the participating centers were not sufficiently acquainted with emergency practice or did not have proper facilities to manage systemic allergic reactions, in view of the severity of many of the reported allergies, confirmative oral challenges (either blinded or open) were carried out only in doubt cases. Coded serum samples were kept at -20°C until in-vitro tests were carried out. 40 randomly selected serum samples out of 64 still available underwent the detection of IgE to arginine kinase and sarcoplasmic calcium binding protein. 28/40 patients had a history of systemic reactions. Nine were sensitized to tropomyosin (rPen a 1), as shown by IgE levels > 0.1 kU/l on ImmunoCAP (ThermoFisher, Uppsala Sweden) (table 1).

Detection of IgE to arginine kinase and sarcoplasmic calcium binding protein

The ISAC 112 Microarray platform (Thermofisher, Phadia, Uppsala, Sweden) was used to detect serum IgE specific for arginine kinase (rPen m 2) and sarcoplasmic calcium-binding protein (rPen m 4) as such allergens are still unavailable in the ImmunoCAP as single allergens. Reactions sites were incubated with 30 µL of patients' serum for 2 hours. After rinsing, washing, and drying, allergen-specific IgE complexes were stained with a fluorescence-labelled anti-human IgE for 30 min. After further washings, a laser scanner took fluorescence readings and results were transformed into numerical data by comparison with a reference serum standardized against ImmunoCAP IgE. As a consequence the results, expressed as ISAC standardized units (ISU/l), are indirectly linked to WHO IRP 75/502 IgE standard. Levels > than 0.3 ISU/l were regarded as positive, following manufacturer's recommendations.

Immunoblot analysis

Patients' sera underwent immunoblot analysis at Lofarma Laboratories, Milan, Italy. Raw shrimp *(Pandalus borealis)* was homogenized and extracted (5%) in 0.1 M phosphate-buffered saline (PBS), pH 7.4, under shaking for 2 hours at 4°C. The protein content, measured after Bradford method, was 1.2 mg/ml. Immunoblots were carried out under reducing conditions. The extract was mixed with LDS sample buffer (40% glycerol and 4% lithium dodecyl sulfate, to prevent adhesion of proteins to glassware and plastic, 4% Ficoll-400, 0.8 M triethanolamine-Cl, pH 7.6, 0.025% phenol red, 0.025% Coomassie G250, 2 mM EDTA disodium (Nupage Bis-Tris, Novex, Life Technologies, Milan) and 5% β−mercaptoethanol. The samples were then denaturated by heating at 100°C for 10 minutes.
Electrophoresis of shrimp extract (25 µg/lane) was carried out in a 10% polyacrilamide precast gel (Nupage Bis-Tris, Novex, Life Technologies) at 180 mA for 1 h. The resolved proteins were transferred for 1 h onto a nitrocellulose membrane according to Towbin et al. (11). The membrane was saturated with 0.1 mol/L tris-buffered saline containing 5% fat-free milk powder and incubated for 16 h at 4°C with sera (dilution 1:1.5 in saturation buffer). After 3 washings, bound specific IgE were detected by peroxidase-conjugated anti-human IgE antibodies from goat (1:4000 in saturation buffer; Biospacific, Emeryville, CA, USA) and using an ECL western blotting kit (GE Healthcare) as substrate.

High molecular weight allergen identification

The high molecular weight allergen protein, detected by the use of a pool of sera reactive on immunoblot analysis (no. 17, 18, 19, 20, 26, 27 in **table 1**; **figure 1**), was identified by N-terminal amino acid sequencing technique. The selected band was excised from SDS-PAGE gel, passively eluted and the N-terminal amino acid sequence analysis was carried out on a Procise 492 protein sequencer (Applied Biosystems, Foster City, CA, USA) as described by Pessione et al. (14). The amino acid sequence was searched using the MS-Homology software at Protein Prospector web site (http://prospector.ucsf.edu/prospector/) against both UniProt KB 2012.03.21 and NCBI nr. 2012.12.3 database. The parameters used were: Order selected Decapoda, no limit for Protein MW, from 1 to 5 possible amino acid changes allowed.

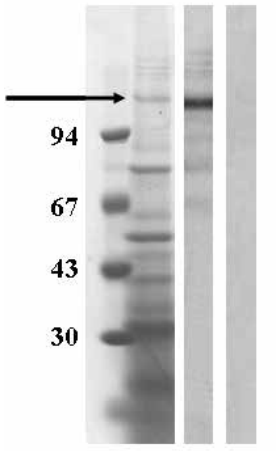

Figure 1 *- Immunoreactive band submitted to N-terminal amino acid sequencing. Lane 1: molecular weight markers; Lane 2: SDS-PAGE of shrimp extract; Lane 3: IgE reactivity to the high molecular shrimp allergen by a pool of sera from patients allergic to both shrimp and mites; Lane 4: negative control serum.*

Ethics

Since this study was an extension of a former one (12) which had been carried out with diagnostic purposes on patients who presented spontaneously in the clinics for clinical evaluation of their shrimp allergy and had already been approved by the local review boards, no further permission by a central ethical committee was required.

Results

IgE to arginine kinase and sarcoplasmic calcium-binding protein

Table 1 summarizes the shrimp-induced allergic reactions experienced by study patients along with the skin reactivity to shrimp and house dust mite and the results of both ImmunoCAP and ISAC assays. A total of 8/40 (20%) patients scored positive for one of the two allergens studied. IgE to rPen m 2 and rPen m 4 were found in 4 (10%) and 6 (15%) cases, respectively; two sera contained IgE against both arginine kinase and sarcoplasmic calcium-binding protein. Although ISAC is only a semi-quantitative analysis, specific IgE levels ranged between 0.8 and 4.0 ISU for rPen m 2, and between 0.6 and 4.2 ISU for rPen m 4. Co-sensitization to arginine kinase and/or sarcoplasmic calcium-binding protein and to tropomyosin was observed only in one case (#35, **table 1**).
Clinically, 6 Pen m 2 and/or Pen m 4 reactors had a history of systemic allergic reactions to shrimp (anaphylaxis in 1 case, urticaria/angioedema in 5 cases), whereas 2 had a history of oral allergy syndrome. Interestingly, anaphylaxis occurred in one patient co-sensitized to both allergens.

House dust mite tropomyosin hypersensitivity

Although the majority of patients (29/38 [76%]) showed a double reactivity to shrimp and house dust mite on skin tests, only 6 (21%) of these reacted to tropomyosin, an allergen that has been considered as the major cause of the cross reactivity between house dust mite and shrimp. Other 6 mite/shrimp reactors were sensitized to either Pen m 2 and/or Pen m 4.
Of the 8 shrimp-allergic patients not sensitized to house dust mite only 1 reacted to tropomyosin and another to Pen m 4.

Immunoblot analysis

Shrimp immunoblot analysis scored positive in 28/40 cases (**figure 2**). Four out of 6 rPen m 4 reactors showed IgE reactivity at about 20 kDa; the two sera scoring negative at this M.W. were those showing the lowest rPen m 4 IgE levels on ISAC microarray (0.8 and 0.6 ISU, respectively). No one of the 4 rPen m 2-positive sera showed IgE reactivity at about 40 kDa on immunoblot analysis; both co-sensitized patients showed an IgE reactivity at 20 kDa only. Nineteen sera showed IgE reactivity at molecular weights > 60 kDa. Such profile was not associated with IgE reactivity to rPen m 2 or rPen m 4.

Identification of the high molecular weight allergen

The sequence obtained by the N-terminal amino acid sequencing matched with a high grade of homology with the hemocyanin subunit 3 from *Homarus americanus* (the American lobster) (**table 2**). Sequences of other hemocyanins from different crustaceans further confirmed our identification.

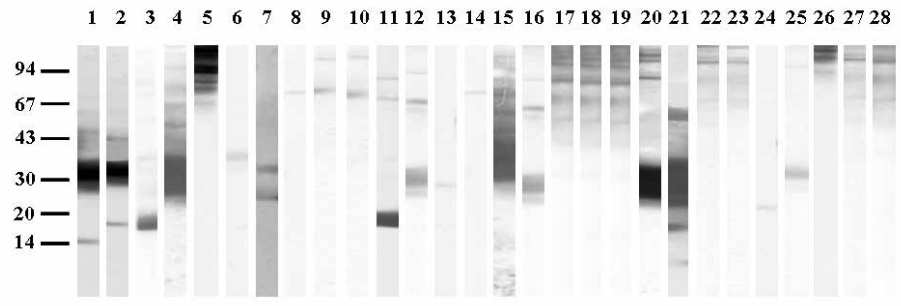

Figure 2 *- Immunoblot analysis of sera from patients 1-28. Patients' numbers correspond to those in table 1.*

Table 2 *- Identification of the immune reacting protein from fig. 2.*

Species	Amino acid sequences	Protein name	UniProt Entry	MW	Homology
Pandalus borealis (our sequence)	[1]NVAQXQHDVNFL			> 100,000 Da	-
Homarus americanus	[9]NVAQKQHDVNFL	Hemocyanin subunit 3 (fragment)	P82298	[1]2,903 Da	92%
Cherax destructor	[7]SDAQKQHDVNYL	Hemocyanin C chain (fragment)	P83172	[1]3,513 Da	67%
Palinurus interruptus	[12]LLAQKQHDVNYL	Hemocyanin C chain	P80096	75,874	67%
Palinurus vulgaris	[6]DNAHKQHDVNHL	Hemocyanin	P80888	75,675	58%

X, amino acid not identified by the N-terminal amino acid sequencing; [1]the database contains only the N-terminal part of the molecules.

Discussion

One of the aims of the present study was to detect the prevalence of hypersensitivity to two recently described shrimp allergens, namely sarcoplasmic calcium binding protein and arginine kinase, in a group of allergic subjects, looking also at their association with tropomyosin sensitization, at their clinical relevance, and at their possible relationship with IgE reactivity against high M.W. shrimp allergens that was frequently observed in our previous study. We demonstrated that both proteins are minor allergens (i.e., recognized by < 50% of the allergic population), as in our population sensitization rate ranges between 10% and 15%. Both sarcoplasmic calcium protein sensitization and arginine kinase sensitization were independent on sensitization to the major allergen, tropomyosin, as only one case of co-sensitization was recorded. Both allergens were found to be clinically relevant as sensitized patients experienced systemic symptoms in 7/8 cases, including one case of anaphylaxis. Finally, our investigations ruled out the possibility that the high molecular weight allergens frequently recognized by Italian shrimp-allergic were actually polymers of Pen m 2 or Pen m 4. The immunoblot assays scored positive at about 20 kDa in most cases of sarcoplasmic calcium-binding protein allergy, whereas no IgE reactivity was observed at about 40 kDa in patients sensitized to arginine kinase. The fact that shrimp extract was heated at 100°C for 10 min might have degraded the heat-sensitive arginine kinase, thus explaining the lack of reactivity at 40 kDa by immunoblotting for sera showing IgE against Pen m 2 in microarray.

The second aim of the present study was to identify the high molecular weight shrimp allergen so frequently recognized by Italian allergic patients. N-terminal amino acid sequencing analysis led to the identification of this allergen as hemocyanin. The first report of hemocyanin as a possible food allergen appeared as long as more than 20 years ago (15). In that study, patients allergic to different sorts of marine gasteropods (limpet and Bolinus brandaris) but also to house dust mite were shown to co-recognize hemocyanin by direct RAST and inhibition assays using commercial hemocyanin from Keyhole limpet (15). In a subsequent study (16), the same group detected a possible involvement of hemocyanin in house dust mite-allergic patients who experienced severe asthmatic reactions following the ingestion of the terrestrial gasteropod snail. Much more recently, a study from Thailand identified hemocyanin as an allergen in a freshwater shrimp (9); in this study, the authors concluded that this allergen is not cross-reactive. Hemocyanin is an oxygen carrier protein representing 75-95% of total proteins in hemolymph of crustaceans (9). In the Decapoda order, hemocyanin is present in the predominant form of hexamers. There are three categories of crustacean hemocyanins called α (1 or A), β (2 or B) and γ (3 or C). We identified the hemocyanin C subunit as an allergen in our shrimp-allergic patients. Some crustacean hemocyanins are demonstrated to be glycosylated (17). Though hemocyanin subunits are reportedly species-specific (18-21), it cannot be excluded that some parts of the protein are phylogenetically conserved and bear allergenic activity. In our previous study (12), we observed that many patients sensitized to high molecular weight shrimp allergens showed house dust mite hypersensitivity as well, and clearly showed that HDM was able to strongly inhibit shrimp IgE reactivity in most cases. This finding is in keeping with that of the older study (15). Further, in a 2001 review article, Sidenius and co-workers (22) stated that "most often, more than one allergen is involved in the HDM snail cross-reactivity in a patient". One further aspect that deserves to be discussed is the different molecular weight of hemocyanin detected by Thai researchers (about 60-80 kDa; Ref 9) and by us (about 100 kDa). It is well known that the molecular weight of homologous proteins in different species may vary and that this could depend on a different degree of glycosilation. In our hands, hemocyanin appeared clinically relevant as

most patients showing predominant reactivity against shrimp allergens > 90 kDa (# 17-20, 22, 23, 26-28 in **figure 2**) had a history of systemic reactions to shrimp, with anaphylaxis in 3 cases (**table 1**).

In conclusion, we suggest that hemocyanin is a clinically relevant shrimp allergen and that it is possibly cross-reactive with shrimp and house dust mite.

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