

IgE Binding to Raw and Boiled Shrimp Proteins in Atopic and Nonatopic Patients with Adverse Reactions to Shrimp

Karen Thursday R. Samson^{a,c} Fen Hua Chen^a Katsushi Miura^a
Yasuhei Odajima^a Yoji Iikura^{at} Maria Naval Rivas^b Kenji Minoguchi^c
Mitsuru Adachi^c

^aDepartment of Pediatrics, Showa University, School of Medicine, Tokyo, Japan, ^bCardinal Santos Medical Center, Manila, Philippines, and ^cFirst Department of Internal Medicine, Showa University, School of Medicine, Tokyo, Japan

Key Words

Shrimp · Adverse reaction · Food allergy · Atopy · IgE

Abstract

Background: Characterization of seafood allergens is important to understand the immune response to these allergens. Moreover, a detailed comparison between atopic and nonatopic patients with adverse reactions to shrimp has never been reported. **Methods:** Raw and boiled shrimp extracts were analyzed by immunoblotting using sera from 9 atopic and 7 nonatopic patients with a history of adverse reactions to shrimp, and 13 control subjects. Total IgE, specific IgE and skin prick tests (SPT) to shrimp were also investigated. **Results:** The level of specific IgE to shrimp was higher in atopic patients than nonatopic patients ($p < 0.05$). Symptoms, SPT results and major allergens involved were similar in atopic and nonatopic patients. The 16.5-kD protein had the highest frequency of IgE binding followed by the 40-kD protein in these patients. Other minor IgE-binding proteins were observed at the 20-, 22-, 54-, 72-, 129- and 140-kD regions. Patients who had binding to the 16.5-kD

protein had either positive (25% raw/31% cooked) or negative (13% raw/cooked) CAP-FEIA-RAST, while patients who recognized the 40-kD protein all had positive (31% raw/19% cooked) CAP-FEIA-RAST. All control subjects had negative immunoblots for these two proteins.

Conclusion: The 16.5-kD protein was the most frequent protein identified regardless of CAP-FEIA-RAST results, while the 40-kD protein was only present in patients with positive CAP-FEIA-RAST. Therefore, 16.5-kD protein may be an important allergen that is clinically relevant in both atopic and nonatopic patients with adverse reactions to shrimp even if it is not detected by the CAP-FEIA-RAST system.

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Introduction

Shrimps are one of the most allergenic seafood species. The increasing demand for seafood led to more reporting of hypersensitivity reactions to it [1–3]. Immediate allergic manifestations to shrimp may involve the skin, gastrointestinal, respiratory and cardiovascular systems.

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Correspondence to: Karen Thursday R. Samson, MD
First Department of Internal Medicine
Showa University, School of Medicine
Hatanodai 1-5-8, Shinagawa-ku, Tokyo 142-8666 (Japan)
Tel. +81 3 3784 8532, Fax +81 3 3784 8742, E-Mail ktrs@med.showa-u.ac.jp

Over the last decade, there has been interest in allergen identification and characterization to further understand the immunopathologic mechanism of food allergy and standardization and quantification of allergens in extracts [4, 5]. Tropomyosin is one of the food allergens that have been well characterized; it accounts for most of the allergenic activity in crustacean allergy. However, identification of other clinically relevant allergens in seafood is still incomplete [6].

Penaeus monodon, popularly known as black tiger shrimp, is widely distributed in Southeast Asia. A number of shrimp allergens, particularly those belonging to *Penaeus* and *Metapenaeus* genera, have already been immunologically identified mostly in atopic patients [7–10]. The major IgE-binding protein, tropomyosin, a 36-kD protein, is also present in other crustaceans, house dust mite (HDM) [11, 12] and cockroach [13]. Other minor IgE-binding proteins with molecular masses ranging from 8 to 166 kD have also been identified but were not well characterized.

The purpose of this study was to evaluate clinically relevant IgE-binding proteins in atopic and nonatopic patients with adverse reactions to shrimp and to investigate their clinical and immunological features.

Materials and Methods

Subjects

Sixteen patients, age ranging from 4 to 53 years old, with a clinical history of adverse reactions to shrimp, characterized as developing symptoms immediately after ingestion of shrimp and requiring medical attention in the emergency room or by a physician, answered a questionnaire eliciting information about past adverse reactions to shrimp, as well as their personal and familial allergic histories. Patients were classified as nonatopic and atopic. Atopy was defined as the presence of clinical symptoms of atopic disease (bronchial asthma, allergic rhinitis and atopic dermatitis) and at least 2 positive parameters of atopy determined in this study (increased serum total IgE and/or positive CAP-IEIA-RAST to more than one environmental allergen) [14]. To assess the severity of the reactions, the symptoms were assigned a weighted score according to Daul et al. [15] with some modifications as follows: generalized pruritus 1; urticaria 2; conjunctivitis (eye itching, eye redness) 3; angioedema 4; gastrointestinal symptoms (nausea, vomiting, diarrhea) 5; upper respiratory symptoms (sneezing, rhinorrhea, nasal congestion) 6; lower respiratory symptoms (shortness of breath, wheezing) 7; generalized systemic anaphylaxis 8. A total score of <12 was classified as a mild reaction, while a total score of ≥ 12 was classified as a severe reaction. Thirteen subjects 4–65 years old, with no history of adverse reactions to crustacea served as control subjects.

Sera were analyzed using the CAP-IEIA-RAST (Pharmacia Diagnostics, Uppsala, Sweden) for measuring IgE reactivity to shrimp, house dust (HD) and HDM. A positive result for CAP-IEIA-RAST was defined as a specific IgE level of ≥ 0.35 UA/ml or class

≥ 1 . Total IgE levels (Pharmacia Diagnostics) were also determined for all subjects. An increased serum total IgE level was based on the manufacturer's reference values.

All subjects gave informed consent before completing the questionnaire and performance of skin prick tests (SPT) and blood collection. This study was approved by the Institutional Review Board of Showa University School of Medicine.

Skin Prick Tests

All subjects had a SPT on the forearm with water-soluble extract of shrimp (Torii Yakuhin, Kobe, Japan) using a bifurcated needle (ALO bifurcated needle, TMI, Tokyo, Japan). The shrimp extract consisted of *P. monodon*, *Penaeus semisulcatus* and *Metapenaeus affinis*. Control prick tests of 1.0 mg/ml histamine (positive control) and saline (negative control) were also applied. An immediate wheal and flare of ≥ 3 mm (mean wheal diameter) was considered as positive. All subjects had wheal and flare reactions to histamine but not to saline.

Shrimp Extract

Black tiger shrimp (*P. monodon*) were purchased from a local supermarket. Raw, heated (60 °C in hot plate for 20 min) and boiled (20 min in ion-depleted water) shrimp meat were used as sources of shrimp antigens. The raw, heated and boiled shrimp muscles were homogenized in 50 ml 0.086 M NaCl solution (Wako Chemicals, Osaka, Japan) containing 0.033 M NaHCO₃ (Nakarai Tesque, Kyoto, Japan) and then extracted overnight at 4 °C with constant stirring. After centrifugation at 12,000 g for 30 min, supernatant was dialyzed using tubes (MW cutoff value of 3 kD) against PBS. The extracts were then aliquoted, frozen and stored at –20 °C. Protein concentrations of the extracts were determined by the method of Bradford using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif., USA).

SDS-PAGE and Immunoblotting

The crude extracts (raw, heated and boiled) were separated by SDS-PAGE as described by Laemmli [16] using 4–20% gradient gel (Asahi, Technos, Tokyo, Japan) under reducing conditions with 2-mercaptoethanol. After electrophoresis, the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Applied Biosystems, Foster, Calif., USA), which were prewashed with methanol and tris(hydroxymethyl)aminomethane (Wako Pure Chemical Industries Ltd., Osaka, Japan)-glycine buffer (Sigma Chemical Co., St. Louis, Mo., USA). The membranes were then cut into strips and washed with PBS containing 0.1% Brij 35 (Sigma Diagnostics, St. Louis, Mo., USA) and blocked with 0.1% human serum albumin (Sigma) in PBS/Brij for 2 h at room temperature. Then membrane strips were washed and serum samples (diluted 1:10 with PBS/Brij) were added and incubated at 4 °C overnight. After washing, membrane strips were incubated with alkaline-phosphatase-conjugated goat antihuman IgE antibody diluted at 1:3,000 (Kirkegaard & Perry Laboratories, Gaithersburg, Md., USA) for 2 h at room temperature. Color development was then carried out using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate solution (Promega, Wisc., USA).

Statistical Analysis

The Wilcoxon/Kruskal-Wallis test was used for comparing non-parametric data (total IgE and RAST) between atopic and nonatopic patients, and patients with adverse reactions to shrimp and control

Table 1. Clinical and laboratory features of patients with adverse reactions to shrimp and control subjects

	Shrimp sensitive		Shrimp nonsensitive	
	atopic n = 9 (56%)	nonatopic n = 7 (44%)	atopic n = 6 (46%)	nonatopic n = 7 (54%)
Sex (male/female)	5/4	0/7	3/3	4/3
Mean age, years	29	35	22	30
Personal history of crustacea				
allergy other than shrimp	6 (67%)	4 (57%)	–	–
Family history of crustacean allergy	8 (56%)	4 (57%)	3 (50%)	0
Symptoms				
Generalized pruritus	5 (56%)	4 (57%)		
Urticaria	8 (89%)	6 (86%)		
Conjunctivitis	3 (33%)	3 (43%)		
Angioedema	3 (33%)	2 (29%)		
Gastrointestinal	0	1 (14%)		
Upper respiratory	3 (33%)	3 (43%)		
Lower respiratory	2 (22%)	4 (57%)		
Systemic anaphylaxis	2 (15%)	0		
Timing of symptoms				
Persistent	5 (56%)	5 (71%)		
Intermittent	4 (44%)	2 (29%)		
Mean onset, min	47	87		
Severity				
Mild	7 (78%)	4 (57%)		
Severe	2 (22%)	3 (43%)		

subjects. Fisher's exact test was used to compare SPT results between patients with adverse reactions to shrimp and control subjects. Spearman's correlation test was used to determine whether there was a correlation between severity of symptoms and total IgE and RAST sensitivity in patients with adverse reactions to shrimp. A p value of less than 0.05 was considered significant.

Results

Clinical Features

We examined 16 patients with a history of adverse reactions to shrimp. Of these, 9 were atopic, while 7 were nonatopic. The symptoms experienced by atopic and nonatopic patients with adverse reactions to shrimp are described in table 1. There were no associations between severity of symptoms and results of total IgE ($r = 0.06$, $p = 0.8$) and between severity of symptoms and level of specific IgE ($r = 0.04$, $p = 0.9$).

Specific IgE Antibodies and SPT Reactivities to Shrimp

Detection of specific IgE antibodies to shrimp gave positive results in 89% of atopic patients and in only 43% of nonatopic patients. The results of CAP-FEIA-RAST

were in agreement with SPT in 63% of cases and the level of specific IgE was significantly higher in atopic patients than in nonatopic patients (4.4 ± 5.4 UA/ml vs. 2.77 ± 5.3 UA/ml; $p < 0.05$, fig. 1a). SPT results were not different between these two groups (fig. 1b). However, those with positive SPT had higher specific IgE antibodies to shrimp compared to those with negative SPT (4.4 ± 5.5 UA/ml vs. 0.51 ± 0.3 UA/ml; $p < 0.001$). The results of the CAP-FEIA-RAST (3.17 ± 5.2 UA/ml vs. 0.70 ± 1.1 UA/ml; $p < 0.01$, fig. 2a) and SPT ($p < 0.0001$, fig. 2b) of patients with adverse reactions to shrimp were significantly different compared with control subjects.

SDS-PAGE of Shrimp Extracts

Raw, heated and boiled shrimp extracts were prepared from different shrimp meat and were used for SDS-PAGE. SDS-PAGE of raw shrimp revealed at least 14 bands with molecular masses ranging from 6.5 to 129 kD. Heated shrimp extract also revealed almost the same bands as raw shrimp but mostly with less dye intensity, except for enhancement of bands at the 20- and 70-kD regions. On the other hand, protein bands less than 40 kD in size were shown in SDS-PAGE of boiled shrimp with enhancement of the 16.5- and 20-kD bands (fig. 3).

Fig. 1. CAP-FEIA-RAST reactivity (a) and SPT reactivity (b) to shrimp in atopic and nonatopic patients. Atopic (n = 9) as compared to nonatopic (n = 7). NS = Nonsignificant difference.

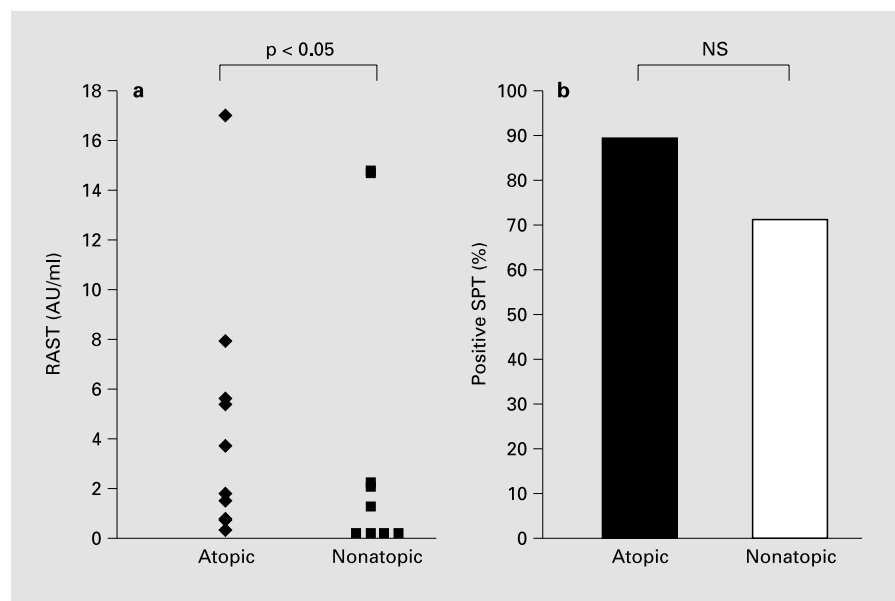
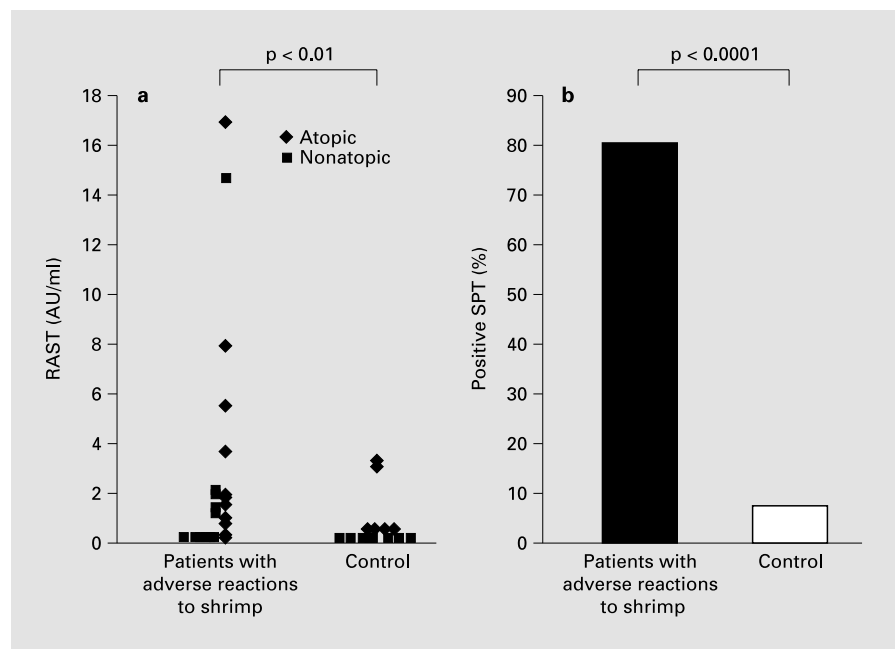


Fig. 2. CAP-FEIA-RAST reactivity (a) and SPT reactivity (b) to shrimp between subjects with adverse reactions to shrimp and those without. Patients with adverse reactions to shrimp (n = 16) compared to control subjects (n = 13).



IgE Reactivities of Subjects' Sera to P. monodon Extracts

To confirm SPT and CAP-FEIA-RAST results, immunoblotting experiments were performed using the raw and boiled shrimp extracts. The majority of the patients exhibited IgE binding at the 16.5- and 40-kD regions (table 2). Other bands were found at the 20-kD (6%), 22-kD (13%), 54-kD (6%), 72-kD (6%), 129-kD (13%) and 140-kD (6%) regions in the raw shrimp extract (fig. 4).

25%/31% (raw/cooked) of the sera of patients with positive CAP-FEIA-RAST recognized the 16.5-kD protein. On the other hand, recognition of the 40-kD protein was seen in 31%/19% (raw/cooked) of patients with positive CAP-FEIA-RAST. Two patients (1 atopic and 1 nonatopic) had IgE binding at the 16.5-kD region although CAP-FEIA-RAST results were negative, while no binding was observed at the 40-kD region in patients with negative CAP-FEIA-RAST.

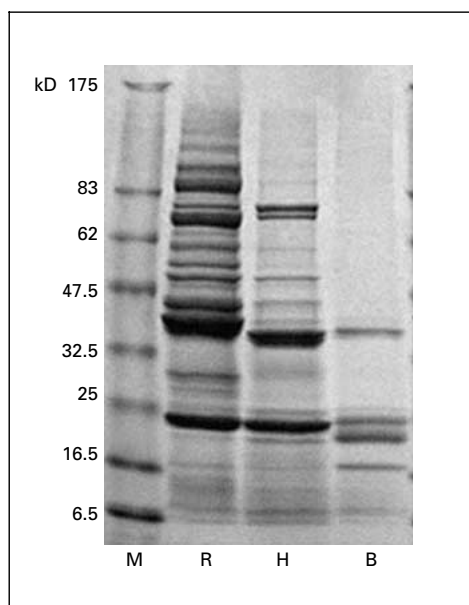


Fig. 3. SDS-PAGE of raw and boiled shrimp (*P. monodon*). Lane M: marker; lane R: raw shrimp extract; lane H: heated shrimp extract; lane B: boiled shrimp extract.

Two atopic control subjects had both positive CAP-FEIA-RAST and IgE binding above the 40-kD region in their immunoblots. The rest of the control subjects had negative immunoblots (fig. 4).

Discussion

We found that atopic patients have a higher level of specific IgE compared with nonatopic patients with adverse reactions to shrimp, while symptoms, SPT results and major allergens involved were similar in these patients. Immunoblotting results demonstrated that not only patients with positive CAP-FEIA-RAST but also those with negative CAP-FEIA-RAST have IgE binding at 16.5 kD in both atopic and nonatopic patients. And that IgE binding at 40 kD was present only in atopic and nonatopic patients with positive CAP-FEIA-RAST. Therefore, atopic patients with adverse reactions to shrimp differed only from nonatopic patients on the level of specific IgE, but the protein that might be responsible for the symptoms is the same and thus the 16.5-kD protein may be a

Table 2. Allergologic features of the 16 patients with adverse reactions to shrimp

Patient	Age/ sex	Status	RAST class	SPT	Western immunoblotting					
					16.5 kD		40 kD		others ¹	
					R	B	R	B	R	B
1	28/F	A	3	+	-	-	-	-	-	-
2	31/M	A	2	-	+	+	-	-	+	-
3	29/F	A	3	+	+	+	+	+	+	-
4	37/M	A	3	+	-	-	+	-	+	-
5	34/F	A	2	-	-	-	-	-	+	-
6	32/M	A	0	+	+	+	-	-	+	-
7	39/M	A	2	+	+	+	+	-	-	-
8	24/F	A	2	+	-	-	-	-	-	-
9	4/M	A	2	+	-	-	+	+	-	-
10	22/F	NA	3	+	+	+	-	-	-	-
11	53/F	NA	0	+	-	-	-	-	-	-
12	36/F	NA	0	+	-	-	-	-	-	-
13	31/F	NA	2	+	-	-	+	+	-	-
14	42/F	NA	0	+	+	+	-	-	-	-
15	28/F	NA	0	-	-	-	-	-	+	-
16	32/F	NA	2	+	-	+	-	-	-	-
Total A					44%	44%	44%	22%		
NA					29%	43%	14%	14%		

NA = Nonatopic; A = atopic; + = positive reaction; - = negative reaction; R = raw; B = boiled.

¹ 20, 22, 54, 72, 129 and 140 kD.

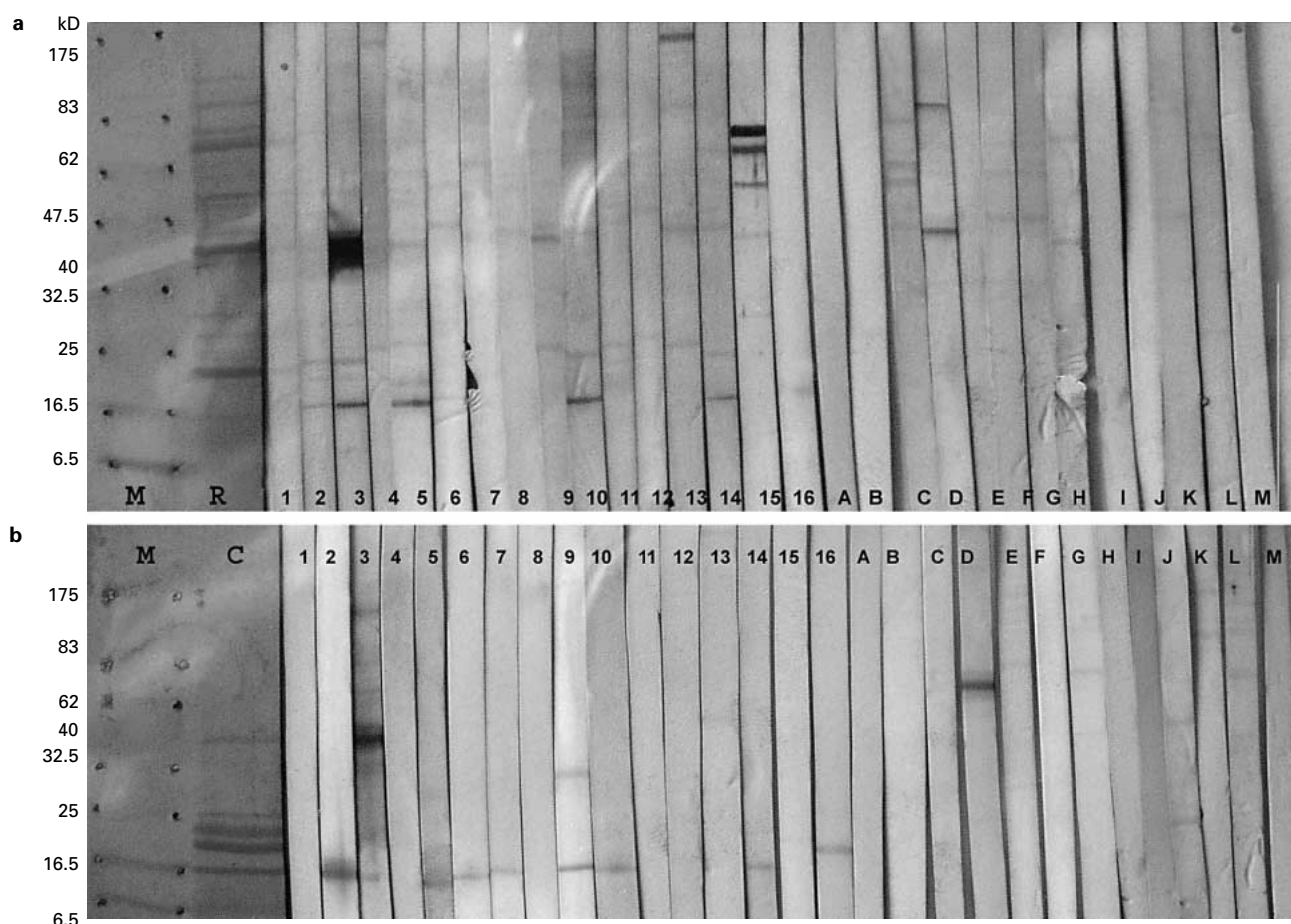


Fig. 4. Immunoblots of *P. monodon* extract. Lane M: molecular mass marker; lane R: Coomassie blue-stained raw shrimp; lane C: Coomassie blue-stained boiled shrimp; lanes 1–9: atopic patients with adverse reactions to shrimp; lanes 10–16: nonatopic patients with adverse reactions to shrimp; lanes A–F: atopic control subjects; lanes G–M: nonatopic control subjects. **a** Raw shrimp extract. **b** Boiled shrimp extract.

clinically relevant allergen in some atopic and nonatopic patients, regardless of the CAP-FEIA-RAST results.

There are only a few studies done in food allergy involving nonatopic patients. Simonato et al. [17] found that the cereal α -amylase inhibitors are major allergens in atopic patients but not in nonatopic patients and that positive immunoblots in nonatopic patients conflicted with the SPT and CAP results. In the present study, symptoms, SPT results and major allergens involved were similar in atopic and nonatopic patients. However, a higher level of specific IgE to shrimp was observed in atopic patients than in nonatopic patients and elevated specific IgE antibodies to shrimp in atopic patients not always concurred with their immunoblot results. In contrast, all nonatopic patients with elevated specific IgE had positive immunoblots. Two atopic patients had positive CAP-FEIA-RAST

but negative immunoblots. This may be explained by the possibility of a species-specific type of hypersensitivity reaction, in which their IgE antibodies against shrimp proteins may be specific for members of the shrimp family other than *P. monodon*. In a previous study, species specificity was reported to be important because it was found to explain intermittent symptoms of reactivity in some patients and that IgE to either unique or shared allergens may explain sensitivity to one or other members of the crustacea class [18]. Meanwhile, 1 atopic and 2 nonatopic patients had negative CAP-FEIA-RAST but showed positive immunoblots. Generally, sera with good IgE reactivity with the CAP system have good reactivity by immunoblotting, although there may be some exceptions to this, since reactivity to a protein was demonstrated despite a negative CAP-FEIA-RAST. This might

be due to the presence of IgE antibodies of low specificity which the CAP system cannot detect but which are sufficient to be detected by immunoblotting, or else the protein that is responsible for reactivity might be absent in the CAP system.

Two atopic control subjects also demonstrated positive results in their CAP-FEIA-RAST and immunoblotting. This raises the issue of atopy as a factor in positivity of specific IgE. In children, there is a high likelihood of false-positive results when atopy is present [19]. Furthermore, increased specific IgE would only indicate the presence of sensitization and does not always equate to clinical reactivity. For the time being, a correlation between the level of specific IgE and prediction of clinical reactivity has been established for egg, milk, peanut and fish [20]. On the other hand, immunoblots of these 2 atopic control subjects did not show bands in the 16.5- and 40-kD proteins, which indicates that they had binding to minor proteins that may not give rise to clinical reactivity.

Tropomyosin is identified as the major shrimp allergen and its IgE-binding epitopes have also been well characterized [21]. Aside from tropomyosin, there are other allergens identified in shrimp with molecular weights of 8–166 kD [9, 10, 22–24]. However, more detailed identification and characterization of these proteins have not yet been performed. Recently, Yu et al. [6] characterized a novel shrimp allergen, Pen m 2 (40 kD), which has an arginine kinase activity different from tropomyosin. Arginine kinase, which has regulatory and/or transport properties, is abundant in shrimp muscle and is also found in other crustaceans; it has been shown to be an important allergen that could also cross-react with other crustaceans in some individuals. In this study, the majority of patients had IgE binding at the 16.5-kD protein followed by the 40-kD protein. Unfortunately, the nature of these two proteins was not further characterized. We did not verify whether the 40-kD protein is arginine kinase. There was other minor IgE binding at the 20-, 22-, 54-, 72-, 129- and 140-kD areas.

Heated shrimp extract revealed the same protein bands on SDS-PAGE as raw shrimp extract, but generally with less dye intensity except for enhancement at the 20- and 70-kD regions. Furthermore, on SDS-PAGE of boiled shrimp, protein bands larger than 40 kD in size were no longer noted as compared with the SDS-PAGE of raw and heated shrimp, but there was enhancement of the 16.5- and 20-kD bands. Proteins become unfolded at temperatures above 55°C with loss of secondary and tertiary structures [25]. The decreased intensity of the bands in heated shrimp and disappearance of bands in boiled

shrimp might be due to loss of some of the protein structures as a consequence of high temperature. Moreover, hydrophobic groups are turned outward and irreversible interactions, which include aggregation or intermolecular interactions, may also occur [26]. It might be possible that high staining intensity was observed because of increased interaction between organic dye and hydrophobic groups of the protein. Also, a strong correlation was shown between the intensity of Coomassie blue staining and interaction between Coomassie dye molecules and basic amino acids, namely, lysine, histidine and arginine [27]. 16.5-, 20- and 70-kD shrimp proteins might also contain a lot of basic charged proteins that became exposed due to unfolding which might have resulted in more interactions with the organic dye molecules.

The effects of thermal processing on egg, milk and milk products have been studied extensively, but only a few have focused on seafood proteins [28–30]. From these studies, thermal processing can lead to the formation of new antigenic structures or loss of some of the original antigenicity of the food protein. Hence, it may be beneficial, but it does not necessarily reduce the risk of allergy to food allergens at all times. On the other hand, heat treatment of peanuts or soybeans neither enhances nor reduces IgE or IgG binding [31]. Changes in the antigenicity of heat-treated food proteins also depend on the individual patient. As observed in our study, 1 nonatopic and 2 atopic patients exhibited binding to the 16.5-kD boiled extract and 40-kD raw extract only, respectively. Moreover, 1 nonatopic patient reported symptoms only after ingestion of raw shrimp but not of cooked shrimp. This patient had IgE binding only to high-molecular-weight proteins (>47.5 kD) in the raw shrimp extract. These findings show that boiling can increase or decrease the allergenicity, depending on individual IgE reactivity to the protein involved.

Studies have found that atopy is a risk factor for systemic anaphylaxis [25, 32]. Another risk factor for anaphylaxis is a personal history of shrimp hypersensitivity reaction [19, 33]. Tropomyosin has been previously identified as the allergen in patients with known anaphylaxis to shrimp [9, 24]. Interestingly in this study, binding to 40 kD alone was observed in patients who had history of generalized systemic anaphylaxis, which was also the second most frequently observed IgE-binding region. Binding to this protein might be a risk factor for developing systemic anaphylaxis.

A limitation of this study is that 16.5- and 40-kD protein were not further characterized. However, because of the high frequency of IgE binding observed in these

regions among the patients, they might be relevant allergens. Further identification and characterization of these two proteins should be done using immunological analysis and proteomics on a larger population.

In conclusion, we have demonstrated that most atopic and nonatopic patients with adverse reactions to shrimp have IgE against the 16.5-kD protein regardless of the specific IgE level, and that IgE binding with the 40-kD protein occurred only in atopic and nonatopic patients with elevated specific IgE to shrimp. Therefore, 16.5 kD might

be an important allergen that is clinically relevant in some atopic and nonatopic patients with adverse reactions to shrimp even if it is not detected by the CAP system.

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