

The use of raw or boiled crustacean extracts for the diagnosis of seafood allergic individuals

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Background: Seafood plays an important role in human nutrition and is responsible for severe hypersensitivity reactions.

Objective: To evaluate how the cooking process may alter the in vivo and in vitro allergenicity of these extracts.

Methods: Raw and boiled extracts of shrimps and 2 types of lobsters were manufactured. Boiled extracts were prepared after the raw material was boiled for 15 minutes in phosphate-buffered saline. Raw and boiled extracts were homogenized and extracted for 4 hours. Afterward, the extracts were centrifuged, dialyzed, filtered, and freeze-dried. Seventy-eight patients were skin prick tested with these raw and boiled extracts. Specific IgE against the 6 extracts was measured by enzyme-linked immunosorbent assay (ELISA). Immunoblots and ELISA inhibition studies were performed with a pool of sera.

Results: In vivo results showed that boiled extracts induced statistically significant larger wheals than raw extracts. More patients with positive results were also detected with boiled extracts. In vitro experiments by direct ELISA confirmed the in vivo results. The protein content in the boiled extracts decreased, and important differences were detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Cross-reactivity experiments showed that both types of extracts retained similar allergenic characteristics, even if the immunoblots revealed some differences in IgE binding.

Conclusions: More patients were identified using boiled extracts of shrimp and American and spiny lobsters than with raw extracts. The wheal sizes of the skin test reactions and specific IgE levels were also significantly greater using boiled extracts. The use of boiled extracts seems to be more effective in diagnosing seafood allergy.

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INTRODUCTION

Seafood is defined as any edible fish or shellfish that comes from the sea. It is composed of different sea organisms, which are important components of the human diet. Shellfish commonly known to cause allergic reactions include shrimp, crab, crayfish, lobster, oyster, clam, scallop, mussel, squid, and snail. These shellfish are important causes of food hypersensitivity and constitute the first cause of food allergy in the American adult population.¹ The most important manifestations include urticaria, angioedema, and acute, potentially fatal anaphylactic reactions and other manifestations that mainly affect the skin and the gastrointestinal and respiratory tracts. Other symptoms, such as dry skin, itching, rash or eczema, chapped skin, and chronic sores, have been described in seafood processing workers.^{2,3} In the United States, the prevalence of seafood allergy is reported at 2.3% of the general population, suggesting that this hypersensitivity represents a significant health concern.⁴ Fish allergies are more common in children, whereas shellfish allergy seems to be more prevalent in adults.⁵

The characterization of seafood allergens is important for better diagnosis of the immune response to these allergens.

Several allergens have been described as major allergens, including tropomyosin, with a molecular weight between 38 and 41 kDa. Tropomyosin is the most common allergen in crustaceans and mollusks and shows great homology in the amino acid sequence among species.⁶ Pen a 1 from *Penaeus aztecus* is the major shrimp allergen, and 75% of all shrimp specific IgE is directed against this allergen.⁷ This protein, which is located in the muscle tissue, has been classified as a panallergen, responsible for the cross-reactivity among crustaceans, insects, and mites.^{8–10} This family of proteins may be useful as a defined and relevant diagnostic marker for allergic sensitization to seafood.¹¹

The importance of the cooking or grilling is well established for most seafood types, and their implications in allergic diseases have been shown. Several studies have suggested that the cooking process may affect the allergenicity of the food.¹² Similar results have been obtained for legumes^{13–16} and mollusks.¹⁷ Therefore, the characterization of raw and boiled seafood extracts is important to select the best allergen extract for the standardization, diagnosis, and treatment of seafood allergic patients. The aim of this study was to evaluate how the cooking process may alter the in vivo and in vitro allergenic capacity of these extracts.

MATERIALS AND METHODS

Extract Preparation

Shrimps (*Penaeus* sp), American lobster (*Homarus* sp), and spiny lobster (*Palinurus* sp) were bought at a local market and used to prepare raw and boiled extracts. The shrimps

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were acquired frozen, whereas both lobster species were alive and killed by freezing at -70°C for 24 hours. Raw extracts were as follows: the seafood was placed in a blender for 3 minutes in 0.01M (1:10 wt/vol) phosphate-buffered saline (PBS). Afterward, the extract was kept under continuous magnetic stirring for 4 hours at 4°C . The boiled extracts were prepared as follows: the seafood was boiled for 15 minutes in 0.01M PBS. Afterward, the content was homogenized and processed as the raw seafood extracts. Subsequently, both raw and boiled extracts were centrifuged, dialyzed overnight against several changes of bidistilled water in 3.5-kDa membranes, sterile filtered, and freeze-dried. The protein content was measured by the Lowry-Biuret method (BioRad Laboratories, Hercules, CA). Sterile skin prick test solutions of both types of extracts were prepared at 5 mg/mL of freeze-dried material in prick solution that contained 50% glycerin, 0.5% phenol, and 0.01M PBS.

Patient Population

The patient population consisted of 78 patients (46 females and 32 males; mean $\pm$ SD age, 34.55 ± 16.7 years) who were skin test positive to at least one of the raw or boiled seafood extracts. The patients resided in Alicante and Murcia (South-east Spain) and had a clinical history of allergic symptoms after the ingestion or contact with seafood. All patients provided written consent.¹⁸ Twenty patients, including 8 non-atopic individuals and 12 atopic patients, who experienced gastrointestinal symptoms or urticaria after the ingestion of various foods were also included as controls and skin tested.

All patients were skin prick tested with the 6 seafood extracts (boiled and raw). Wheal sizes were measured and compared using the Wilcoxon signed rank test. The results were expressed in millimeters squared and considered positive when the reaction was 7 mm² or higher. A serum sample was collected for further in vitro analyses. After the specific IgE determinations were completed, a serum pool was prepared with the serum of 20 patients.

Specific IgE Determination

Specific IgE to the 6 different seafood extracts (raw and boiled) was determined by direct enzyme-linked immunosorbent assay (ELISA). Plastic microtiter plates were coated with a final concentration of 20 $\mu\text{g/mL}$ of freeze-dried material. The final concentration per well was 2 μg , corresponding to a mean protein concentration of 1 μg . Each serum was diluted 1:2 (vol/vol) in 0.01M PBS and incubated (100 μL) for 2 hours in the wells. The plates were washed and incubated for 2 hours with monoclonal α -human IgE (Ingenasa, Madrid, Spain) conjugated with peroxidase (2 ng/mL). After 5 washes, the reaction was developed for 30 minutes, using a solution that contained tetramethyl-benzidine, hydrogen peroxide, and water and stopped with 1N sulfuric acid. Three individual serum samples from nonallergic patients were used as negative control. The result was considered positive when a serum bound 3 times more specific IgE than the mean of a negative control.

Protein and Allergenic Profiles

The protein profile of the seafood extracts was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Raw and boiled extracts were loaded onto 15%T, 2.67%C gels under reducing conditions. Gels were stained with Coomassie blue (BioRad).

The protein profile of the extracts was determined by immunoblot experiments using a pool of sera to determine differences in allergen recognition. After electrophoresis, the gels were transferred onto Immobilon membranes (Millipore, Bedford, MA) and incubated overnight with a pool of sera diluted 1:5 in PBS 0.1% Tween. After washing, the membranes were incubated with peroxidase-conjugated anti-human IgE (Ingenasa, Madrid, Spain).

ELISA Inhibition Experiments

A serum pool was prepared with serum aliquots of 20 patients. Plastic microtiter plates (Immulon IV, Dynex Technologies, Chantilly, VA) were coated with a raw shrimp extract (10 $\mu\text{g/mL}$ of freeze-dried material) overnight. Several dilutions were made from the extracts. Each dilution was incubated with the serum pool for 2 hours at room temperature. Afterward, 100 μL was transferred to the coated plates and incubated overnight. After washing, 100 μL of anti-human IgE peroxidase was added and let to stand for 30 minutes at room temperature. After washing, the plates were developed for 30 minutes and stopped with 1N sulfuric acid.

Statistical Analysis

The statistical analysis consisted of descriptive and nonparametric statistics (Wilcoxon signed rank test). The prevalence of positive skin test reactions to boiled and raw extracts was compared by contingency tables (χ^2). Results were considered statistically significant at $P \leq .05$.

RESULTS

Protein Content

Total protein contents of the seafood extracts were as follows: raw shrimp, 519 $\mu\text{g/mg}$; boiled shrimp, 437 $\mu\text{g/mg}$; raw American lobster, 702 $\mu\text{g/mg}$; boiled American lobster, 474 $\mu\text{g/mg}$; raw spiny lobster, 591 $\mu\text{g/mg}$; and boiled spiny lobster, 564 $\mu\text{g/mg}$. In all cases the protein content decreased after the extracts were boiled.

Patient Population and Skin Prick Test Results

Enrolled patients had a positive skin test result to at least 1 of the 6 seafood extracts; 9 were sensitized to 1, 16 to 2, 15 to 3, 13 to 4, 5 to 5, and 20 to 6. Other sensitivities were also evaluated; 53% were sensitized to mites, 35% to different pollens, 12% to epithelia, and 14% to cockroach. The most frequently reported symptoms after seafood ingestion were angioedema (46%), urticaria (24%), and oral allergy syndrome (20%). Four patients (5%) had experienced anaphylaxis after prawn ingestion. Eighteen patients, who were skin test positive to at least 1 extract, were asymptomatic after the ingestion of these 3 crustaceans.

Nine of these patients were positive to all the extracts, 5 to 2 extracts and 4 to 1. The serum of 20 of these patients was selected for further in vitro assays (Immunoblot and ELISA inhibition). Eight (40%) of these 20 patients were skin test positive against the 6 extracts, 2 (10%) to 5, 5 (25%) to 4, 1 (5%) to 3, and 4 (20%) to 2. Three (15%) of these patients had oral allergy syndrome, 10 (50%) had angioedema, 6 (30%) had urticaria, and 5 (25%) tolerated the ingestion of seafood.

Boiled extracts induced a greater percentage of positive skin test reactions than raw extracts; 56 patients (72%) were positive with the raw prawn extract vs 59 (76%) with the boiled extract ($P = .58$). The raw American lobster extract was positive in 47 patients (60%) vs 61 (78%) with the boiled extract ($P = .02$). The raw spiny lobster extract was positive in 40 patients (51%) vs 55 (70%) with the boiled extract ($P = .01$).

The mean of the wheal sizes induced with boiled prawn, American lobster, and spiny lobster extracts was significantly greater than when raw extracts were used ($P = .003$, $P = .004$, and $P = .03$, respectively, Wilcoxon rank test). A box plot view of the wheal sizes is shown in Figure 1. The mean $\pm$ SD skin test size for the raw prawn was 19.12 ± 17.77 vs 30.14 ± 32.55 for the boiled extract, 26.4 ± 28.65 for the raw American lobster vs 34.75 ± 36.33 for the boiled extract, and 21.41 ± 23.84 for the raw spiny lobster vs 26.73 ± 21.71 for the boiled extract. All control individuals had negative skin test results to all raw and boiled extracts.

Protein and Allergenic Profiles

The SDS-PAGE gel of the seafood extracts is presented in Figure 2. In all cases, the protein profile was modified after

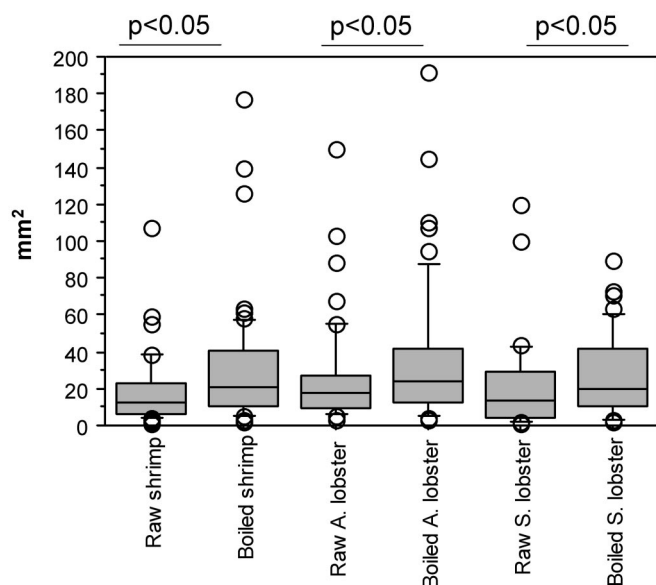


Figure 1. Box plot view of the skin test results induced by raw and boiled extract in 78 allergic patients. In all cases, there was a significant difference between the raw and the respective boiled extracts.

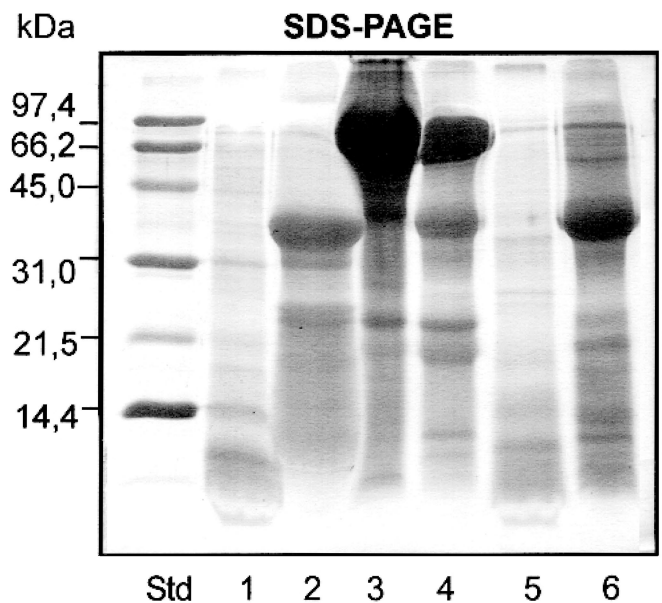


Figure 2. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel of the seafood extracts. Std, Molecular weight standards; lane 1, raw shrimp; lane 2, boiled shrimp; lane 3, raw American lobster; lane 4, boiled American lobster; lane 5, raw spiny lobster; and lane 6, boiled spiny lobster.

the extracts were boiled. The changes observed were greater for the shrimp and spiny lobster than for the American lobster extracts. The detection of a band with an approximate molecular weight of 38 kDa was enhanced in all boiled extracts.

The immunoblot experiments revealed the presence of several allergenic proteins. Certain differences were detected between boiled and raw extracts in their allergenic profiles after immunoblots (Fig 3). Higher-molecular-weight bands were seen in the boiled samples, with an approximate molecular weight of 90 kDa, which could correspond to aggregation products. These proteins were also visible, although to a lesser extent, in the raw extracts. IgE binding to these high-molecular-weight bands was detected for raw and boiled American lobsters and for the boiled spiny lobster. After boiling, all these extracts retained their specific IgE binding capacity, especially to the 38- to 40-kDa allergen, which could correspond to tropomyosin. The exact nature of the high-molecular-weight specific IgE binding bands, especially in boiled extracts, remains to be established, since these molecules have not been characterized.

Specific IgE Determinations

The optical density values of specific IgE for each extract are shown in Figure 4. Statistically significant differences were found between raw and boiled extracts ($P < .001$). The percentages of sera with a positive specific IgE determination to raw vs boiled extracts were as follows: prawn, 23.6% vs 49.1%; American lobster, 30.9% vs 40%; and spiny lobster, 41.8% vs 78.2%.

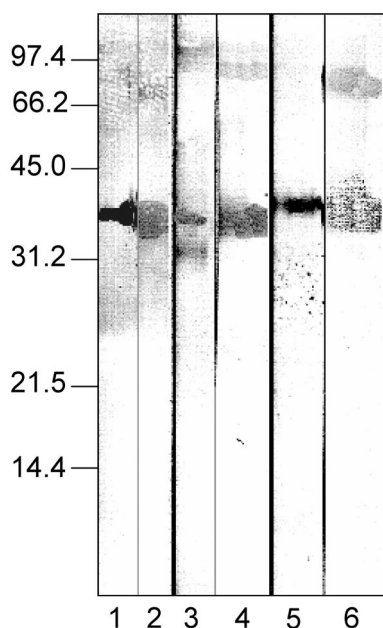


Figure 3. Immunoblot. Lane 1, Raw shrimp extract; lane 2, boiled shrimp extract; lane 3, raw American lobster; lane 4, boiled American lobster; lane 5, raw spiny lobster; and lane 6, boiled spiny lobster.

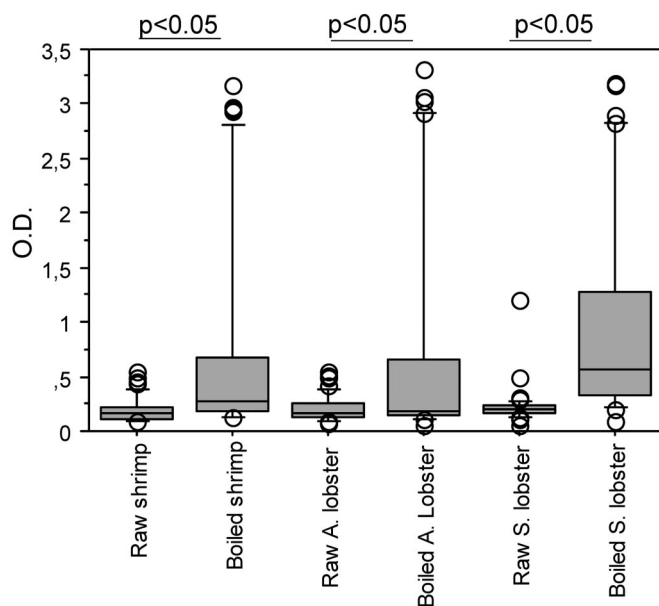


Figure 4. Box plot view of specific IgE levels to raw and boiled extracts. O.D. indicates optical density.

ELISA Inhibition Experiments

Figure 5 shows the results of the ELISA inhibition experiments to determine cross-reactivity among the analyzed crustaceans extracts. Using the raw shrimp extract in the solid phase, all curves remained within the limits of parallelism

when compared with the raw shrimp extract, suggesting a comparable allergenic composition. All inhibition values were similar, suggesting a high degree of cross-reactivity. The amounts of protein to produce the 50% inhibition points were as follows: raw shrimp, 0.349 μg ; boiled shrimp, 0.303 μg ; raw American lobster, 0.534 μg ; boiled American lobster, 0.301 μg ; raw spiny lobster, 0.46 μg ; and boiled spiny lobster, 0.361 μg .

DISCUSSION

Hypersensitivity reactions to crustacean allergens are an important cause of allergic hypersensitivity reactions worldwide. Sensitization may occur through ingestion or inhalation,^{19–21} although most adverse reactions occur after consumption.²² Our study evaluated 78 patients who showed allergic symptoms after seafood ingestion. All of them had clinical manifestations with the ingestion of different types of seafood (bivalves, cephalopods, and mollusks).

The existence of allergens that are resistant to the heat treatment has been previously described.^{23–25} However, high temperatures, such as those reached during cooking or grilling, tend to break the disulfide bridges of proteins, modifying their secondary and tertiary structures and their capacity to bind antibodies.²⁶ In these cases, the protein profile of the extracts can also be modified, producing extracts with a different protein profile. Furthermore, the allergenicity can also be reduced or enhanced because of the creation of neoallergens.²⁷ The production of neoallergens as a consequence of heat or storage has been described in different extracts.^{28–31}

In the present study, we demonstrated that when raw and boiled extracts were compared, protein bands, which were present in the raw extracts, were no longer visible in the boiled extracts. However, new bands were also formed. This finding could be explained by the fragmentation of the proteins by the effect of the temperature, inducing the formation of different fragments, or peptides, with lower molecular weights. The cooking process induced the appearance of new bands in the molecular weight range of 25 to 45 kDa. In all cases, the most important characteristic of these boiled extracts was the presence of a prominent band with a molecular weight of approximately 40 kDa, which was not clearly visualized in the raw extracts. Other allergens were present in the molecular weight range of 40 to 80 kDa. Similar results were described by different authors, who reported IgE binding to a 40-kDa band.³² Other allergens with 16.5-, 20-, 22-, 54-, 72-, and 129-kDa bands have also been demonstrated.¹²

To determine the *in vivo* allergenicity of these extracts, all patients were skin prick tested with raw and boiled extracts. The results obtained demonstrated that boiled extracts induced larger wheal sizes than raw extracts. We were able to detect 4% more patients sensitized to shrimps, 18% more to American lobster, and 19% more to spiny lobster. These data suggest that heated extracts are significantly more effective to diagnose sensitization to American and spiny lobsters by skin prick tests.

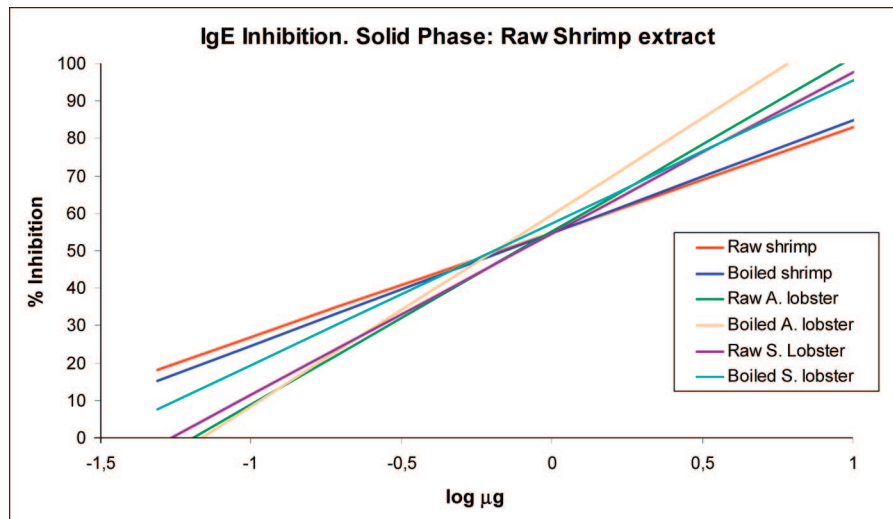


Figure 5. Enzyme-linked immunosorbent assay inhibition experiments comparing the 6 extracts using a raw shrimp extract in the solid phase. A. indicates American; S., spiny.

Certain differences were observed in specific IgE binding after Western blots of raw and boiled extracts. The higher-molecular-weight bands that appear in boiled samples in Figure 3, especially for the spiny lobster, may play a role in the observation that boiled extracts bind significantly more specific IgE than raw extracts. Breakdown products, too small to detect in a Western blot, could also account for these differences.

Specific IgE determinations confirmed the differences between both types of extracts. In all cases, optical density was significantly higher for boiled than for raw extracts. We can therefore corroborate that boiled prick extracts have a greater capacity to detect sensitized patients, which is in agreement with the consumption of these seafood after heating. It seems that patients are mostly sensitized to thermostable or heat-modified allergens present in boiled extracts. According to these results, we can speculate that patients become sensitized to heat-modified proteins, especially in Spain, where the ingestion of raw crustaceans is uncommon.

Cross-reactivity among different shellfish species is well established.³³ More than 80% of the prawn sensitized population have a positive skin prick test result or an immediate reaction after the ingestion of other crustaceans.³⁴ In the present study, cross-reactivity between raw and boiled extracts was evaluated using a serum pool of sensitized patients. In all cases, the 50% inhibition point was very similar, indicating a high degree of similarity in the allergenic composition of these extracts.

In summary, we have analyzed the immunochemical characteristics of 3 raw and boiled crustacean extracts. We conclude that the boiling process significantly alters the protein profile of the crustacean extracts, as evaluated by SDS-PAGE. It also modifies the in vivo and in vitro allergenic activity of these extracts. Molecular changes and the fact that

patients have only been in contact with boiled shrimp and American and spiny lobster allergens may explain the greater wheal sizes induced in vivo and the specific IgE levels detected in vitro. Our study suggests the importance of using the correct diagnostic extract to diagnose seafood allergy and warrants standardization of these extracts. Boiled extracts seem to be better than raw extracts to diagnose seafood allergy in our population.

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