



Analytical Methods

Simulated gastrointestinal digestion reduces the allergic reactivity of shrimp extract proteins and tropomyosin



Cristina Gámez^{a,b}, M^a Paz Zafra^{a,b}, Verónica Sanz^{a,b}, Carla Mazzeo^{a,b}, M^a Dolores Ibáñez^c, Joaquín Sastre^{b,d}, Victoria del Pozo^{a,b,*}

^aImmunology Department, IIS-Fundación Jiménez Díaz, Spain

^bCIBER de Enfermedades Respiratorias (CIBERES), Spain

^cAllergy Department, Hospital Niño Jesús, Spain

^dAllergy Department, IIS-Fundación Jiménez Díaz, Spain

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ABSTRACT

Shrimp are highly allergenic foods. Current management are limited to the avoidance of foods. Therefore, there is an unmet need for a safe and effective therapy using modified allergens. This study focuses on assessing the potential for modification of the allergenicity of shrimp proteins following heat treatment or simulated gastric digestion. Shrimp proteins do not reduce their IgE reactivity after heat treatment but it is reduced by simulated gastric digestion in a time- and dose-dependent manner. Tropomyosin in shrimp extract is worse digested than purified tropomyosin. After 60 min of 10 U/μg pepsin digestion, a strong inhibition was produced in the *in vivo* skin reactivity of shrimp extracts and in activation of basophils from allergic patients. Immunisation experiments performed in rabbits demonstrated that digested boiled shrimp extract is able to induce IgG antibodies that block the IgE binding to the untreated boiled shrimp extract in shrimp-allergic patients.

Building on our observations, digestion treatment could be an effective method for reducing shrimp allergenicity while maintaining the immunogenicity.

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1. Introduction

Shrimp allergy is a common cause of allergic reaction worldwide (Lopata & Lehrer, 2009). It may induce mild to severe allergic reactions (Albrecht et al., 2008) and usually persists throughout life. In spite of the high prevalence of shellfish allergy, few options are available for treatment, and avoidance of the offending food is the recommended therapy. New treatments of oral immunotherapy with milk, eggs or peanut have obtained good

previous results, but they still have some limitations (Jones, Burks, & Dupont, 2014).

Currently, several crustacean allergens have been characterised: arginine kinase (García-Orozco, Aispuro-Hernández, Yepiz-Plascencia, Calderón-de-la-Barca, & Sotelo-Mundo, 2007), sarcoplasmic calcium-binding protein (Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008), myosin light chain (Ayuso et al., 2008), troponin C and triosephosphate isomerase (Bauermeister et al., 2011). However, the muscle protein tropomyosin is the major allergen identified in shrimp. This 37 kDa protein is associated with the actin filament of muscle cells as well as other non-contractile cells. Presence of specific IgE to tropomyosin has been demonstrated to be an effective marker of clinical reactivity to shrimp (Gámez et al., 2011; Yang et al., 2010). Moreover, patients with clinical shrimp allergy show more intense binding to tropomyosin peptides and greater epitope diversity (Ayuso et al., 2010). Different shrimp tropomyosins have been identified from several types of shrimp, Pen a 1 in brown shrimp, Pen m 1 in black tiger shrimp, Lit v 1 from white leg Pacific shrimp, Pen j 1 from Kuruma prawn, and Met e 1 from greasyback shrimp or sand shrimp. Their amino acid sequence identity ranges from 93% to

Abbreviations: SGF, simulated gastric fluid; DBPCFC, double blind placebo control food challenge; SPT, skin prick test; RSE, raw shrimp extract; BSE, boiled shrimp extract; PBS, phosphate-buffered saline; TM, tropomyosin; ME, mercaptoethanol; SAV-HRP, streptavidin horseradish peroxidase; ECL, enhanced chemiluminescence; DBSE, digested boiled shrimp extract; BAT, basophil activation test; HRP, horseradish peroxidase; ODI, optical density results with the rabbits' immune sera; ODP, optical density results with the rabbits' preimmune sera; DTM, digested tropomyosin.

* Corresponding author at: Immunology Dept., Fundación Jiménez Díaz, Av. Reyes Católicos 2, 28040 Madrid, Spain. Tel.: +34 91 5504891.

E-mail address: vpozo@fdj.es (V. del Pozo).

99%. Tropomyosins from dust mites and other arthropods show sequence identity of about 75–80% (Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002).

Although the mechanisms by which individuals become sensitised to food allergens are unclear, most food allergen sensitisation may occur via the gastrointestinal tract (Bannon, 2004). Several studies report that tropomyosin is an extremely stable protein that retains (Leung et al., 1994) and even increases (Carnés et al., 2007) its allergenic activity after heat treatment.

However, the stability and allergenicity variation of tropomyosin and other shrimp proteins to simulated gastric fluid (SGF) are not well known (Kunimoto et al., 2011; Schnell & Herman, 2009).

This study focuses on assessing modifications of the allergenicity of tropomyosin and other shrimp proteins after simulated gastric fluid digestion.

2. Materials and methods

2.1. Patient selection

A total of 36 patients with a history of immediate symptoms after ingestion of shrimp were recruited from the Allergy Departments of two hospitals (Fundación Jiménez Díaz and Hospital Niño Jesús, Madrid, Spain). Some were also sensitised to mites. Shrimp allergy was assessed by double-blind placebo-controlled food challenge (DBPCFC). The clinical characteristics and results of DBPCFC have been previously reported (Gámez et al., 2011). The study was approved by the hospitals' ethics committees and all patients/guardians provided prior informed consent in writing.

Sera and blood samples were obtained from all patients. A pool of sera from selected patients was used for IgE immunodetection. Among all the patients, only five patients and one non-allergic subject signed the consent to test modified extracts by skin prick test (SPT).

2.2. Preparation of shrimp extracts

Solenocera melanthero was used for preparations of raw (RSE) and boiled shrimp extracts (BSE). Boiled shrimp was prepared by heating at 60 °C for 10 min in phosphate-buffered saline (PBS) (1:3, w/v). RSE and BSE were prepared as described previously by Ayuso et al. (2008). The protein concentration of each extract was determined using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA, USA).

2.3. Purification of shrimp tropomyosin

The purification of shrimp tropomyosin (TM) was carried out as described by Kunimoto et al. (2009) with some modifications. Raw shrimp was minced and homogenised in 2 volumes of extraction buffer A (50 mmol/l KCl, 2 mM NaHCO₃, 10 mmol/l 2 mercaptoethanol (ME, pH 8.0).

The homogenate was centrifuged at 12,000×g, for 20 min at 4 °C. The precipitate was collected and suspended in 2 volumes of extraction buffer A. After 3 repeated cycles of homogenisation and centrifugation, the resulting precipitate was dissolved in 99.5% ethanol (1:2, w/p) and passed through a nylon filter (Beckton Dickinson, San José, CA, USA). The precipitate was washed with 4 volumes of 100% ethanol and 2 volumes of diethyl ether and dried overnight. The powder obtained was suspended in extraction buffer B (20 mmol/l Tris-HCl, 1 mol/l KCl, 10 mmol/l 2ME) and stirred overnight at 4 °C. The extract was clarified by centrifugation at 12,000×g for 20 min at 4 °C in Beckman Centrifuge J2-21M/E (Beckman Coulter, Fullerton, CA, EEUU). The supernatant was adjusted to pH 4.5 with 1 mol/l HCl and allowed to stand for 1 h

at room temperature. After another centrifugation, the precipitate obtained was dissolved in 1 mmol/l NaHCO₃, and the solution was adjusted to pH 7.5 with 1 mol/l KOH and allowed to stand for 1 h at room temperature, and centrifuged again. Finally, 2ME and KCl were added to the supernatant, yielding a final concentration of 10 mmol/l and 1 mol/l, respectively. The isoelectric precipitation process was performed twice more.

Successively, the supernatant was partially purified by ammonium sulphate fractionation at 50% saturation. The pellet was collected by centrifugation at 12,000×g for 15 min at 4 °C and dissolved in 1 mmol/l NaHCO₃ pH 7.5 and then dialyzed against bdH₂O.

The protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA, USA).

2.4. Digestibility of purified TM and shrimp extract proteins in SGF

For this purpose, the allergens were incubated with pepsin (0.1 and 10 U/μg protein) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) as a standard SGF (2% (w/v) NaCl, 0.7% (v/v) HCl, pH 1.2), and preheated at 37 °C for 15 min before adding the tested proteins. Digestion was performed at 37 °C with continuous shaking. An aliquot (100 μl) of the digest was withdrawn at different time intervals (0, 5, 30 and 60 min). Digestions were stopped by quick addition of 1 mol/l TRIS.

2.5. SDS-PAGE analysis and immunoblotting

Raw and boiled shrimp extract proteins and purified TM were separated by SDS-PAGE under reducing conditions as described previously (Luengo et al., 2008).

For immunodetection of IgE-binding proteins, the separated proteins were electroblotted onto a nitrocellulose membrane (BioRad, Richmond, CA, USA) and incubated for 18 h at 4 °C with 1/3 dilution of different pool sera or individual sera. Bound IgE antibodies were detected using biotinylated conjugated rabbit anti-human IgE (INGENASA, Madrid, Spain) follow by SAV-HRP (Beckton Dickinson, San José, CA, USA) using the ECL chemiluminescence method as recommended by the manufacturer (Amersham Biosciences, Buckinghamshire, UK).

2.6. Skin prick tests

SPTs with a commercial shrimp extract (Leti, Madrid, Spain) and prick-by-prick tests with raw red shrimp (*S. melanthero*) were performed according to European guidelines (EAACI Subcommittee on Skin Tests, 1993). Also, undigested boiled shrimp extract (BSE), digested boiled shrimp extract (DBSE), and SGF were tested in 6 subjects (5 patients and 1 non-allergic volunteer). Histamine phosphate (10 mg/ml) and saline buffer were used as positive and negative controls, respectively. To compare SPT results, we measured wheal areas (mm²).

2.7. Basophil activation test (BAT)

BSE and DBSE were tested at 1–40 μg/ml for activation of basophils in 3 whole blood samples from shrimp-allergic donors and 1 non-allergic individual. This test is based on analysis of CD203 up-regulation on IgE-positive basophils by flow cytometry using BasoFlowEx[®]Kit (EXBIO Diagnostics, Huissen, Netherlands). The tests were considered positive when the percentages of activated basophils surpassed the cut-off value of 15%.

2.8. Immunisation of rabbits

Rabbits were immunised with 600 µg of BSE or DBSE using complete Freud adjuvant, followed by booster injections of 600 µg of extracts using incomplete Freud adjuvant, and one last booster of 800 µg (boosters were given with a one-week interval).

Titration of rabbit IgG raised against BSE and DBSE to ELISA plate-bound BSE proteins (5 µg/ml) was performed with serial dilutions (1/100 to 1/100,000) of rabbit antisera. Shrimp-specific IgG antibodies were detected using HRP-labelled goat anti-rabbit IgG (Merck Millipore, Darmstadt, Germany).

All experimental procedures were carried out in strict accordance with the international and national (Real Decreto 1201/2005) guidelines and were approved by the Fundación Jiménez Díaz Animal Research Ethics Committee. All protocols were performed under conditions to minimise animal suffering.

2.9. Inhibition of allergic patients' IgE binding to BSE by polyclonal antiserum against DBSE

The ability of DBSE and BSE to produce rabbit IgG with IgE blocking activity in allergy patients was tested by ELISA competition assay (Vrtala et al., 1998).

ELISA plates (Costar 3590, Corning Incorporated, NY, USA) were coated with BSE (5 µg/well) and pre-incubated with 1/1000 dilution of the BSE and DBSE rabbit antisera, and, for control purposes, the pre-immune sera. After washing, plates were incubated with 1/3 diluted patients' sera. Bound IgE antibodies were detected with goat anti-human IgE-HRP (Sigma Chemical Co., St. Louis, MO, USA) diluted 1/5000.

The percentage of inhibition of IgE binding achieved by pre-incubation with the anti-BSE and anti-DBSE antisera was calculated as follows:

$$\text{Inhibition (\%)} = 100 \times (1 - (\text{ODI}/\text{ODP}))$$

ODI and ODP are the Optical Density results with the rabbits' immune sera and pre-immune sera pre-incubation, respectively.

2.10. Statistical analysis

Data comparisons between the untreated or digested shrimp extracts were performed using the Mann–Whitney *U* test and an ANOVA unpaired *t*-test with Welch's correction for nonparametric data and parametric data, respectively. Normality was analysed using the Kolmogorov–Smirnov test. Differences were considered to be significant when $P \leq 0.05$. Statistical analyses were performed using Graphpad Instant 3 software.

3. Results

3.1. Digestibility of RSE and BSE by SGF

RSE and BSE were examined for digestibility by SDS–PAGE. As shown in Fig. 1A and B, the digested protein profile of RSE and BSE were similar. In SGF reactions, shrimp proteins began to be digested during the 5 first minutes, even at a ratio of 0.1 U pepsin/µg of protein. A protein band around 42–45 kDa disappeared within the first seconds of treatment (lane 0, Fig. 1) and 90, 20 and 14 kDa proteins were undetected within 5 min. In contrast, TM was not completely digested under 0.1 U/µg SGF conditions (Fig. 1A and B).

Interestingly, when 10 U pepsin/µg of protein was used, all shrimp proteins were degraded after 60 min of treatment, although a slight TM band can be observed in Fig. 1A.

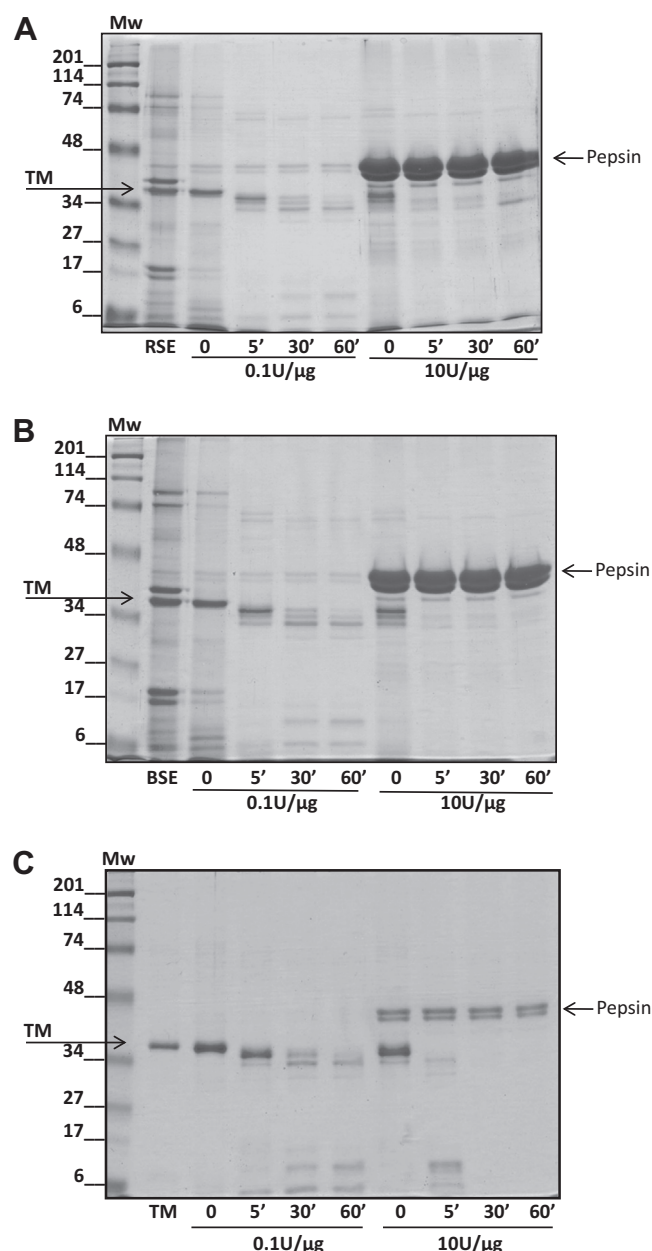


Fig. 1. Effect of pepsin digestion on shrimp extracts. SDS–PAGE of shrimp extract: raw (A), and boiled (B); and on purified tropomyosin (C) after pepsin digestion (0.1 U/µg and 10 U/µg of protein). The content of loaded proteins from shrimp extracts and purified tropomyosin were 10 µg and 2.5 µg, respectively. The protein in SDS–PAGE was stained with colloidal Coomassie blue. RSE: raw shrimp extract, BSE: boiled shrimp extract; TM: tropomyosin; Mw: molecular weight markers.

3.2. Digestibility of purified TM

The result of purified TM *in vitro* digestion is shown in Fig. 1C. Purified TM revealed a band corresponding to the molecular mass of 37 kDa (Fig. 1C). Digestive patterns of TM by pepsin at 0.1 U or 10 U were quite different. TM was more stable in SGF at a ratio of 0.1 U/µg, even after 60 min of treatment; faint fragments around 34, 15, and 5 kDa were detected (Fig. 1C). After 30 min of treatment, no TM fragment bands were detected (Fig. 1C) when the digestion ratio used was 10 U/µg.

3.3. IgE reactivity of untreated and digested shrimp extracts

The IgE-binding ability of digested RSE (Fig. 2A) and BSE (Fig. 2B) was evaluated by immunoblotting using a sera pool from

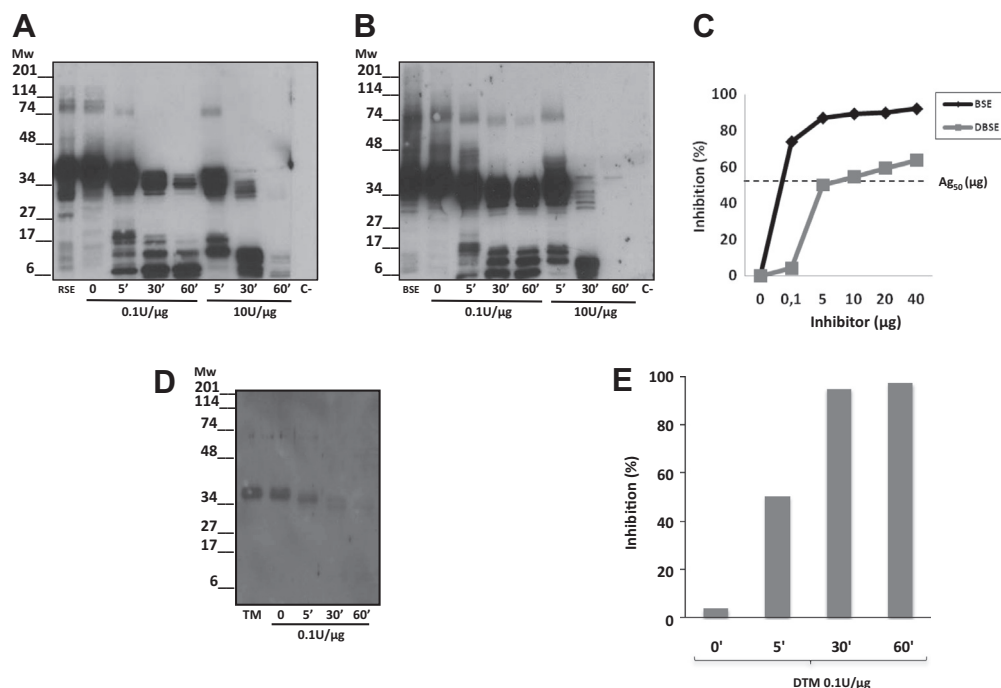


Fig. 2. Effect of digestion on IgE reactivity. IgE reactivity of untreated (RSE) and digested raw shrimp extract (DRSE) (A); untreated (BSE) and digested boiled shrimp extract and (DBSE) (B) were performed by immunoblotting using a sera pool from shrimp-allergic patients. (C) ELISA inhibition of IgE binding to BSE with undigested BSE (black) and DBSE (grey) using sera pool from shrimp-allergic patients. Ag_{50} : antigen concentration needed for 50% inhibition. (D) IgE reactivity of purified tropomyosin (TM) before and after pepsin digestion (DTM) were performed by immunoblotting using serum from an allergic patient. (E) Bands were quantified by densitometry and histogram show the % of IgE inhibition to digested tropomyosin at different times. Mw: molecular weight markers.

shrimp allergic patients. As shown in Fig. 2A and B, digestion reduced substantially the IgE reactivity of RSE and BSE. High molecular weight RSE digested fragments, which appeared after 30 min of 0.1 U/μg treatment, were not IgE reactive. TM IgE-binding capacity was reduced after 60 min of 0.1 U/μg treatment, but did not disappear completely, because smaller fragments were detected with IgE from the pooled sera. When 10 U/μg protein was used, IgE reactivity of RSE proteins was almost eliminated after 60 min treatment, including IgE reactivity to TM (Fig. 2A).

In contrast, when BSE was digested (Fig. 2B) at 0.1 U pepsin/μg protein, high molecular weight fragments continue to be IgE reactive, even after 60 min of treatment. In the presence of 10 U pepsin/μg of BSE proteins, 60 min of treatment made TM unable to bind to IgE. Because maximal inhibition of IgE-binding was observed under these conditions, 10 U pepsin/μg of protein for 60 min was chosen as the optimal treatment for subsequent experiments.

The non-allergic donor showed no IgE reactivity to RSE, BSE, or to the digestion products (data not shown).

To confirm the diminution of DBSE IgE reactivity, we performed ELISA inhibition experiments. As can be observed, 0.07 μg of BSE was able to inhibit 50% of IgE binding from pool sera while 5 μg of DBSE was necessary to reach the same inhibition (Fig. 2C).

3.4. IgE-binding capacity of untreated and digested purified tropomyosin

The IgE-binding capacity of purified TM by pepsin digestion was analysed by IgE immunoblotting (Fig. 2D). Western blotting with sera from one shrimp-allergic patient showed that IgE antibodies reacted with the untreated TM. IgE reactivity of purified TM was completely reduced after 60 min of digestion at a ratio of 0.1 U/μg of protein (Fig. 2D).

Densitometry analysis verified the reduction of IgE-binding capacity to digested TM. As shown in Fig. 2E, when digested purified TM (DTM) was treated with pepsin (0.1 U/μg) for 60 min, IgE-binding capacity was almost eliminated (97.4% inhibition).

The non-allergic donor showed no IgE reactivity to untreated purified TM or DTM (data not shown).

3.5. Allergenic activity of BSE and DBSE by *in vivo* and *ex vivo* assays (SPTs and BAT)

The allergenicity of BSE and DBSE was compared by *ex vivo* and *in vivo* tests.

The *in vivo* allergenic activity was studied by SPTs using shrimp-allergic patients. BSE induced immediate skin reactions, whereas most patients showed no reaction or mild reactions when tested with DBSE after 60 min of treatment (10 U/μg of protein) (Table 1).

When we compared the reduction in SPT areas of BSE versus DBSE after 60 min of digestion, we observed a significant difference ($81.5 \pm 29\%$, $P < 0.05$) (Fig. 3A). An example of SPTs from one

Table 1
Effect of digestion on the *in vivo* skin reactivity of shrimp extracts.

	Subjects					
	1	2	3	4	5	6
Prick prick	15.9 ^a	23.7	95	78.5	50.3	0
Shrimp prick	0	19.6	0	44.2	63.6	0
BSE	56.7	15.9	78.5	122.7	33.2	0
DBSE ^b 0'	28.3	70.1	28.3	71	0	0
DBSE 60'	4.9	0	7.1	86.6	0	0
Pepsin 0'	0	0	0	0	0	0
Pepsin 60'	0	0	0	0	0	0
Histamine	33.2	1.9	86.6	78.5	33.2	0

^a Wheal area (mm²).

^b Digested with 10 U pepsin/μg protein.

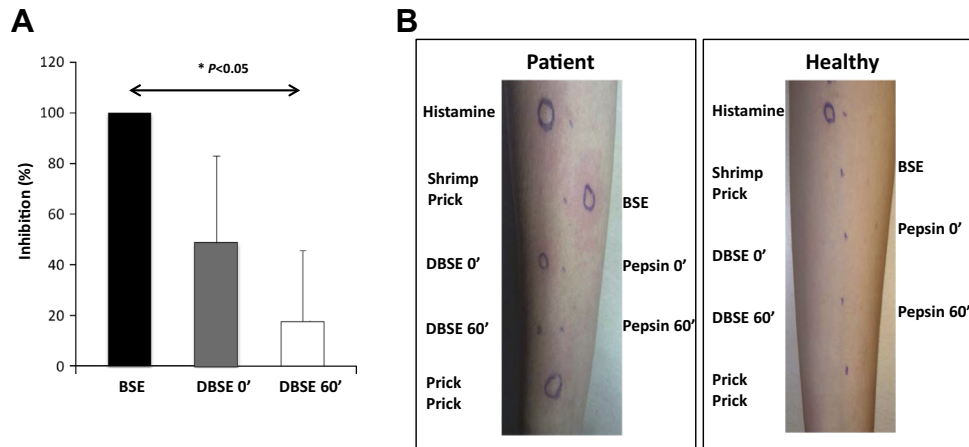


Fig. 3. Effect of digestion on SPT. (A) Reduction in SPT areas of boiled shrimp extract versus digested boiled shrimp extract after 0 and 60 min of treatment (10 U pepsin/ μ g of protein). (B) Representative picture of SPTs from one shrimp-allergic patient and one non-allergic individual (healthy). BSE: boiled shrimp extract. DBSE: digested boiled shrimp extract (0 and 60 min of treatment). Pepsin: negative control of digestion (0 and 60 min of treatment).

shrimp-allergic patient and one non-allergic donor are shown in Fig. 3B.

Pepsin in SGF was also tested, and no reaction was detected in any patient (Table 1). The non-allergic subject showed no reactions to any of the used extracts (Fig. 3B).

The allergenic activity of BSE and DBSE was also analysed by basophil activation test, measuring CD203c up-regulation in blood basophils from three shrimp allergic patients and the non-allergic subject on exposure to various amounts of the shrimp extracts (Fig. 4). As can be observed, there was an up-regulation of CD203c expression after incubation with BSE at the maximum concentration (from 72.1% up to 92.1%; Fig. 4). When basophils were

incubated with DBSE at the maximum concentration, the CD203c expression was reduced significantly in all patients (from 37.2% up to 50.8%). No CD203c expression was detected at any concentration of BSE and DBSE on blood basophils from non-allergic donor (Fig. 4).

Pepsin-SGF did not produce CD203c expression in any subject (Fig. 4).

3.6. IgG antibodies induced by immunisation with DBSE inhibit patients' IgE binding to BSE

We examined whether BSE- and DBSE-induced rabbit IgG antibodies could inhibit the binding of allergic patients' IgE to BSE. For

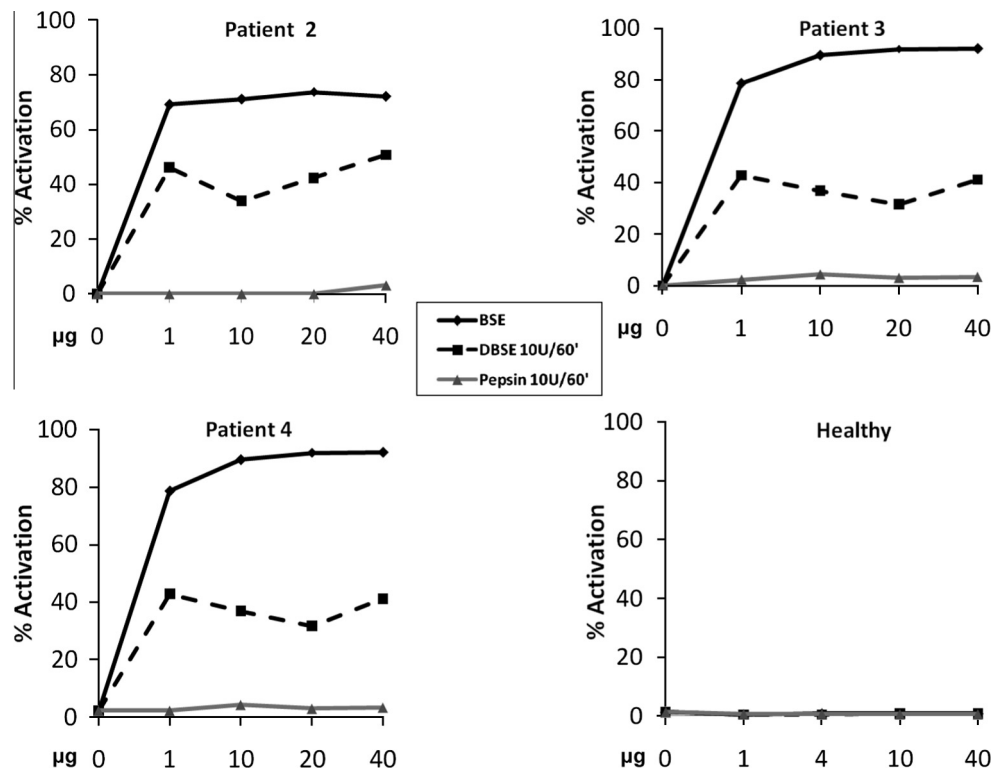


Fig. 4. Effect of digestion on basophil activation. Basophil activation induced by undigested (continuous line) and digested (discontinuous line) boiled shrimp extracts from 3 shrimp-allergic patients and one non-allergic donor. Pepsin used as negative control (Pepsin 10 U/60'). Cells were stimulated with increased amount of proteins (x-axes). Basophil activation percentage is displayed at the y-axes. BSE: boiled shrimp extract; DBSE 10 U/60': digested boiled shrimp extract with 10 U pepsin/ μ g of protein during 60 min.

Table 2
Inhibition of allergic patients' IgE binding to DBSE by rabbit IgG antibodies.

Patient No.	Inhibitor	
	rαBSE	rαDBSE
1	100 ^a	11.6
2	100	7.5
3	100	16.5
4	66.5	6
5	55.3	5.2
6	100	11.7
7	100	34.3
8	100	33.4
9	100	58.3
10	100	48.1
11	46	4.9
12	100	85
13	56.8	8.9
14	100	29.3
Mean ± SD	87.5 ± 21	25.8 ± 24 ^b

^a % Inhibition.

^b $P < 0.0001$.

this purpose, ELISA competition experiments were performed with sera from 14 shrimp-allergic patients. Pre-incubation of BSE with rabbit IgG raised against BSE antibodies reached an inhibition percentage that ranged between 46% and 100% with a mean inhibition of $87.5 \pm 21\%$ (Table 2). Interestingly, rabbit anti-DBSE inhibited patients' IgE binding to BSE between 4.9% and 85% with a $25.7 \pm 24\%$ mean inhibition (Table 2).

4. Discussion

In our study, we proved that after simulated gastric fluid digestion, the allergenicity of tropomyosin and other shrimp proteins were reduced considerably. This finding was demonstrated not only by diminished IgE binding being, but also by SPT and BAT. These *in vivo* and *ex vivo* techniques have not been used previously in studies of shrimp allergenicity after simulated gastrointestinal digestion.

We choose *S. melanthero* because is one of the most consumed shrimp worldwide, but our results with this species could be extrapolate to other shrimp because the allergic proteins have high sequence homology; for example, tropomyosins from several shrimp species have sequence similarity ranging from 93% to 99%.

During digestion, food proteins are subjected to different types of processing, such as heat treatment, acidification, and hydrolysis. These significantly affect their structures and consequently their resistance to digestion. Furthermore, shrimp in Europe are often consumed after cooking, so we examined the effect of boiling on protein profile and the allergenicity. We observed that boiling did not alter protein profile significantly, but was associated with greater IgE binding to high-molecular-weight proteins (Figs. 1 and 2). Several authors have reported findings that support our data (Carnés et al., 2007; Mine & Yang, 2008), specifically that BSE has enhanced specific IgE binding more than RSE. The explanation for this phenomenon could be that protein aggregation occurred during heat treatment or some epitopes are more accessible after boiling. High temperatures achieved during cooking break disulphide bridges of proteins, modifying their secondary and tertiary structures, and also their capacity to bind to IgE with the subsequent creation of new allergens (Poms & Anklam, 2004).

In contrast, Liu et al. (2010) maintained that boiling of shrimp extract caused lower IgE binding. However, purified TM from boiled shrimp enhanced IgE binding over that of raw shrimp.

In this work, we studied the stability and allergenicity of *S. melanthero* extract and purified TM after gastric digestion. We show

for the first time that digested *S. melanthero* proteins from RSE and BSE exhibit considerably reduced IgE-binding capacity (Figs. 1 and 2). These findings are in contrast to those obtained by others (Liu et al., 2011) probably due of the different conditions of digestion. Thus, when the pepsin/protein ratio was increased, IgE binding to TM underwent a notable decrease (Huang et al., 2010).

It is widely acknowledged that human digestion is variable from person to person, so it was not easy to choose an optimal pepsin-to-substrate ratio because pepsin activity depends on pH and other physiological differences (Hur, Lim, Decker, & McClements, 2011; Moreno, 2007). In order to perform *in vitro* digestion experiments that reproduce physiological conditions in the gastrointestinal tract, we employed standard SGF as recommended by American Pharmacopeia (Ph US), and the ratios used were 0.1 U/μg and 10 U/μg.

For digestion experiments, many authors use purified proteins in order to assess their allergenicity, but this approximation may be less accurate due to the presence of other food proteins in *in vivo* digestion. This could be the reason why purified TM seems to have reduced stability compared with TM in shrimp extract. For this reason, in subsequent experiments, BSE was employed.

Since IgE-binding capacity is not directly equivalent to allergenicity, it is necessary to perform *in vivo* functional experiments such as basophil activation capacity and SPTs (Huang et al., 2010; Liu et al., 2011).

Until now, functional experiments with DBSE have not been conducted. SPT wheal areas using DBSE were smaller than those obtained with BSE. Commercial shrimp prick tests produced smaller wheals than BSE (Table 1), and it could be important to standardise the prick used because boiled crustacean extracts are more effective for allergy diagnosis, as has been suggested by Carnés et al. (2007). Furthermore, the results from BAT demonstrated how digestion treatment reduces the adverse effects associated with the IgE-mediated activation of basophils. Similarly, Vassilopoulou et al. (2006) demonstrated the reduction of IgE-binding capacity and histamine release of digested lipid transfer protein (LTP) from grape.

It is important to note that immunotherapy of food allergies is not a common course of treatment because, among other reasons, potent allergenic foods tend to induce several adverse reactions (Nelson, Lahr, Rule, Bock, & Leung, 1997). However, reducing or eliminating IgE antibodies binding capacity, inducing allergen-specific IgG antibodies, and preserving T cell reactivity by allergen modification are currently under discussion as promising new strategies for food allergy treatment (Reese et al., 2005). In this sense, we would expect that "vaccination" of shrimp-allergic patients with DBSE would induce allergen-specific IgG antibodies, which block allergen-induced effector-cell degranulation and IgE-mediated inflammation. Immunisation experiments performed in rabbits demonstrated that DBSE is able to produce IgG antibodies, which block the IgE binding to the BSE in shrimp-allergic patients. We also found that digestion with pepsin preserved T-cell epitopes during T-cell proliferation *in vitro* using cells from three shrimp-allergic patients (data not shown).

Similar results have been reported with genetically modified allergen derivatives (Ball et al., 2009).

In conclusion, we have demonstrated that purified TM and shrimp extracts do not retain their IgE-binding capacity or their allergenic potency after gastrointestinal digestion. The present study also demonstrates for the first time and by *in vivo* and *ex vivo* assays that shrimp allergenicity can be reduced. Building on this observation, digestion treatment could be a good approach to reduce shrimp allergenicity in order to obtain a safe and effective "vaccine" for shrimp allergy.

Conflict of interest

None of the authors has any potential financial conflict of interest related to the manuscript.

All authors have read and agreed to its content, and that any experimental research that is reported in the manuscript has been performed with the approval of an appropriate ethics committee (56017/001).

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