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Evaluation of the sensitivity of Moroccans to shrimp tropomyosin and effect of heating and enzymatic treatments

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ABSTRACT

The aim of this work was to evaluate the IgE-sensitivity to shrimp tropomyosin (ST) in a Moroccan population from Fez region, and then to study the effect of temperature and enzymatic digestion on the allergenicity of ST. This work was conducted with a questionnaire completed by a sera-bank, obtained from 500 patients recruited from Fez Hospitals. Their sera were analyzed for specific IgE-sensitivity to ST. From questionnaire, 9.8% reported allergy to fish and shellfish where shrimp was one of the most common species causing allergy in patients. Evaluation of specific IgE showed that 10.2% of patients present higher values. Further indirect ELISA and Dot-blot results indicated that ST showed a decrease in the human IgE binding under heating or pepsin hydrolysis. These results demonstrate that this population was sensitive to ST and the sensitivity could be reduced by heating and more where it was digested by pepsin.

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Fish/shellfish allergy; shrimp tropomyosin; human IgE; heating; enzymatic digestion

Introduction

Fish and shellfish are important foods in the world including Morocco. However, these products induce important allergic reactions in both children and adults. Among allergic shellfish, shrimp is one of the most common allergens worldwide due to their widespread consumption, especially in coastal countries (Daul, Slattery, Reese, & Lehrer, 1994; Lopata & Lehrer, 2009; Priftis et al., 2008; Schäfer et al., 2001; Steensma, 2003). The estimated prevalence of shellfish allergy reported in numerous countries was less than 18% (Kim et al., 2008; Rona et al., 2007; Sánchez & Sánchez, 2015; Zuberbier et al., 2004). However, in Morocco, no study is focused on shellfish sensitivity or shellfish consumption.

Research has demonstrated that the major allergen of shellfish was Tropomyosin, a myofibrillar protein of about 35–38 KDa (Gámez et al., 2015; Lopata & Lehrer, 2009; Zhenxing, Hong, Limin, & Jamil, 2007). At least 80% of individuals with shrimp allergy react with this major allergen, which is bound to approximately 85% of shrimp-specific IgE from shrimp-allergy subjects (Leung et al., 1994). Moreover, the 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).

The efficacy of food processing techniques, such as heating and enzyme hydrolysis, to reduce food allergenicity, has been previously tested. Their effects may increase or decrease the allergenicity of proteins, depending on the severity of the treatment (Bousfiha & Aarab, 2013; Cabanillas et al., 2012; Ma et al., 2015; Ouahidi, El Hamsas, & Aarab, 2011; Wang et al., 2014; Xu, Shi, Yao, Jiang, & Luo, 2016).

The aim of this study was first to evaluate the prevalence of self-reported fish/shellfish allergy and the IgE-sensitivity to ST in a Moroccan population from Fez region, and then to study the effect of temperature and enzymatic digestion on the allergenicity of ST.

Material and methods

Patient's sera and sample collection

The study was conducted at the University Hospital Centre of Fez and at Ibn El Khatib-Hospital in order to collect serum samples. Sera were obtained from 500 patients with question whether they had allergic reactions to food and fish/shellfish in particular. Patients were asked to provide information on the species they tried, the presence of allergic symptoms and types of fish and shellfish allergy (sardine, mackerel, shrimp, squid, common Pandora, whiting, etc.). It should be noted that they were chosen at random. They were people who came to different medical tests. This work was conducted from May 2014 to June 2015 and was approved by the Ethic committee of the University Hospital Center of Fez.

After formal consent of the patients, a blood sample of 3 ml was collected in a dry tube. After centrifugation at 3000 rpm during 5 min, sera were separated and stored at -20°C until use.

Extraction and purification of ST

ST was separated and purified as previously described (Asturias et al., 1999; Li, Lin, Cao, & Jameel, 2006), with some modifications. Briefly, shrimp muscle (5 g) was incubated in 50 ml of extraction buffer (1 mol/L KCl and 0.5 mmol/L β -mercaptoethanol, pH 7.0) for 16 h at room temperature, and centrifuged at $12,000\times g$ for 15 min. The precipitate was extracted one more time for 2 h. The pooled supernatants were cooled to 4°C . Further purification was obtained after precipitation the supernatant with 35–60% ammonium sulfate saturation. After centrifugation at 6000 rpm for 20 min, the precipitate proteins solution was applied to gel filtration on a Sephadex G-100 column, which was eluted with 0.01 mol/L phosphate buffer (pH 7.0). Fifty fractions (2 ml per tube) were collected and measured for absorbance at 280 nm. Then, the Purified protein solution was concentrated against 10% PEG (Polyethylene glycol) and used for experiments or stored at -20°C until used. Quality of extracted proteins was evaluated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Elisa analysis

Specific IgE to purified ST was determined by indirect ELISA as previously described (Bousfiha & Aarab, 2013; Ouahidi et al., 2011). Briefly, 100 μl of ST (0.5 mg/ml) was

deposited per well in 96 well microplates and incubated for 60 min at 37°C. Then, 200 µl of 0.5% bovine serum albumin (BSA) was added to every well for an hour at 37°C. After removal of BSA, human sera were added (100 µl/well) before incubation with Goat anti human IgE peroxidase conjugate for 60 min at 37°C. Bending of anti-IgE was revealed by adding 100 µl of 0.05% OPD (orthophenylenediamine). The reaction was stopped by adding HCl 3 M. Then the developed color was measured by absorbance at 490 nm.

SDS-PAGE and dot-blot assay

Extracted ST was separated by SDS-PAGE (Denaturing protein electrophoresis). Samples (100 µl per well) were mixed with loading buffer (10% SDS, 10% glycerol, 10% β-mercaptoethanol, and 2.5% bromphenol blue), heated at 100°C for 5 min, and electrophoresed in 15% analytical SDS-polyacrylamide gels. After migration, the gel was stained with Coomassie Brilliant Blue R-250.

Dot-blot assay was performed as previously described (Cai et al., 2010; Zheng, Lin, Pawar, Li, & Li, 2011) with some modifications. In brief, purified ST was spotted on nitrocellulose (NC) membranes (5 µl for each dot) and left to dry at room temperature 37°C. The spotted membranes were saturated in borate buffered saline (BBS) Tween (2.5%) for 1 h at 37°C to block the non-specific binding sites. After washing, the membranes were incubated with human sera overnight. After incubation with anti-IgE peroxidase conjugate, the reaction was revealed by the incubation of membranes in a solution containing 0.05% of diaminobenzidine (DAB) in BBS tampon. The intensity of spot indicates the reactivity of specific IgE to ST.

Effect of heating and enzymatic digestion

Purified ST (0.5 mg/ml) was exposed to different temperatures (70°C, 80°C and 90°C) for various times (30, 60 and 120 min). For enzymatic treatment, ST was digested with pepsin (30 µg/ml) in an acid environment (pH 2) for 30, 60 and 120 min at 37°C. Thereafter, ST treated was deposited in 96 well microplate and human IgE binding evaluated by ELISA and Dot-blot assay as described before.

Preparation of rabbit anti-ST antibodies

To study the immunoreactivity of IgG antibodies to ST, Rabbit IgG was prepared against native ST. These antibodies were obtained after repetitive immunization of rabbits against the native tropomyosin using Freund adjuvants as described before (Bousfiha & Aarab, 2013; Ouahidi et al., 2011). After one month, animals were sacrificed, blood samples were collected in dry tubes and sera separated. Then, sodium azide 0.1% was added to the sera and frozen at -20°C until used.

Statistical analysis

Descriptive statistics were presented as numbers with percentages or as average values. Statistical analysis was based on the student's *t*-test taking $p < .05$ as the limit of the significant value.

Results

Patients

A total of 500 patients were selected and surveyed in the University Hospital Centre of Fez and in Ibn El Khatib- Hospital of Fez. The sample was composed of 300 females (60%) and 200 males (40%). There were 76.6% patients, represented by Adults, aged between 20 and 60 years, and 23.4%, represented by children, aged between 1 and 20 years.

Reported adverse food reactions

In our study, the self-reported food adverse reaction, in these patients, was 21.4% ($n = 107$). It was mostly associated with fish and shellfish (9.8%), followed by milk (8.2%) and cereals (3.4%). From the 9.8% ($n = 49$) who reported fish/shellfish sensitivity, 53% had multiple fish sensitivities. As regard age, self-reported fish/shellfish sensitivity was most pronounced in adults (12%) than in children group (2.6%). According to sex, the self-reported fish/shellfish sensitivity was most marked in male (13.5%) than in female (7.3%). Prevalence according to fish species was highest for sardine, reported by 87.7% ($n = 43$) followed by mackerel with 42.8% ($n = 21$), and shrimps with 22.4% ($n = 11$).

Regarding the results of self-reported shrimp sensitivity, we noted that prevalence was higher in adults (82%) than in children (18%). From this population, 64% had multiple fish sensitivities and 82% reported of cooked and fried shrimp sensitivity. The most frequent clinical signs were cutaneous reactions (55%) and gastrointestinal reactions (36%).

Specific IgE measurement

Sera of the 500 patients have been tested for specific IgE binding to ST. The average of positive values (>2 IU/ml) was 95.9 IU/ml ($n = 215$) varying between 2.75 and 323.58 IU/ml. Dosage of specific IgE has shown that 16.2% of patients present values higher than 100 IU/ml and 10.2% concerning the values higher than 150 IU/ml. According to age and sex, the results showed a lower prevalence in children (6%) than adults (11.5%) and in female (9.3%) than in male (11.5%) population with a rate higher than 150 IU/ml.

SDS-PAGE of ST

Extracted ST was analyzed by SDS-PAGE (Figure 1). Tropomyosin appeared as a band corresponding to the molecular mass nearly 36–38 kDa. Under temperature at 90°C, SDS-PAGE profile indicated a decrease in the intensity of the ST band after one hour of treatment. By the effect of digestion processing, we noted that the intensity of ST band, treated with pepsin, was lower than that with heating effect. When ST was exposed to both treatments (heating followed by pepsin hydrolysis), bands became less pronounced and intensity partially disappeared.

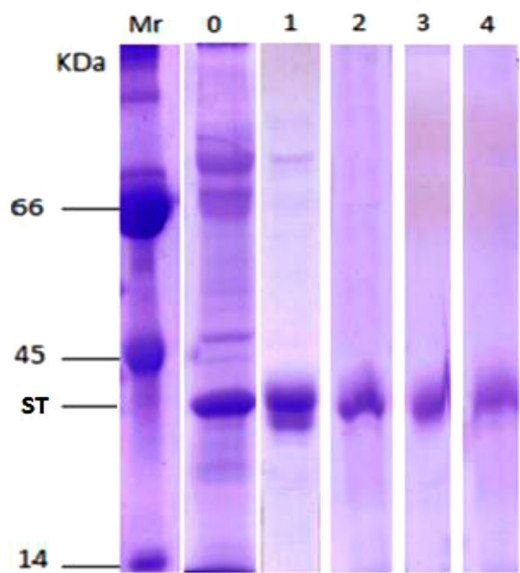


Figure 1. SDS-PAGE analysis of Shrimp tropomyosin. Mr, markers of the molecular weights; 0, Native ST before precipitation by ammonium sulfate; 1, Native ST after precipitation by ammonium sulfate; 2, ST treated by temperature; 3, ST treated by pepsin; 4, ST treated by the combination of two treatments.

Effect of heating and enzymatic digestion on the detection of ST by rabbit IgG using ELISA and dot-blot assays

The variation in immunoreactivity of ST after heating and enzymatic digestion was firstly assessed by using rabbit IgG anti-ST. The aim was to determine the parameters of reduction of the ST-binding to specific antibodies by using ELISA and Dot-blot.

Results (Figure 2) showed that the IgG binding to ST was decreased at 80°C after 30 min of treatment and was highly diminished at 90°C. Concerning the effect of enzymatic treatment (Figure 3), we noticed that protein pepsin hydrolysis altered the binding of IgG to ST. The maximum reduction observed was 90.5% with pepsin, 87.8% with heating and 85.1% with combined effects. These reductions are not statistically different.

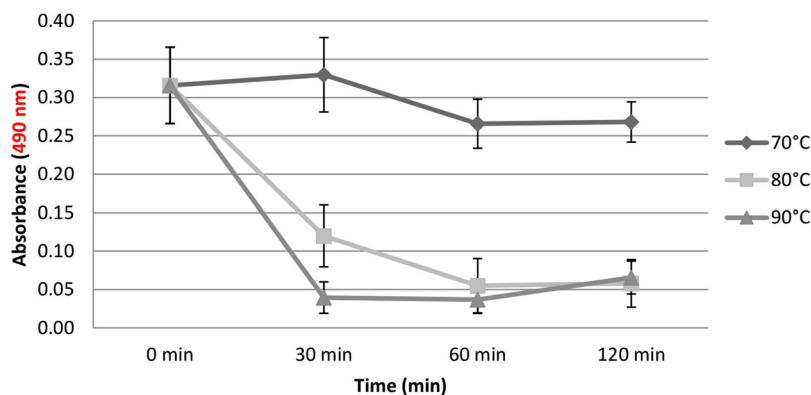


Figure 2. Effect of different temperatures on the recognition of ST by rabbit IgG.

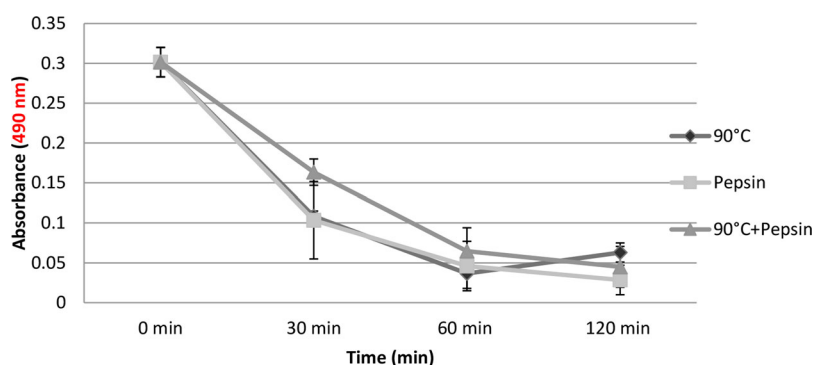


Figure 3. Effect of heating and enzymatic digestion on the recognition of ST by rabbit IgG.

This decrease in reactivity was confirmed by Dot-blot analysis (Figure 4). The Dot-blot assay achieved by the anti-ST rabbit IgG showed the presence of a spot corresponding to tropomyosin, indicating the reactivity of rabbit IgG towards ST. After treatments, we noted that heating at 90°C reduced the intensity of blotting spot, while enzymatic digestion processing (during one hour) has almost removed this reactivity.

Effect of heating and enzymatic treatments on human IgE binding to ST using ELISA and dot-blot assays

Human sera of 20 patients with higher specific ST-IgE levels (>100 IU/ml) were selected and used to estimate the binding variation of IgE to ST under temperature at 90°C and enzymatic treatment (during one hour) using ELISA (Figure 5) and Dot-blot assays (Figure 6). Under heating at 90°C, we observed that all patients ($N = 20$) showed a decrease in the IgE binding to ST from 21.8% to 84.1% with an average diminution of 56.7%. Where protein was treated by pepsin (Figure 5), we noted for all patients tested a reduction in IgE recognition varying from 67.3% to 99% with an average of 89.4%. When the two treatments were combined (heating and pepsin), we noted an average diminution in the IgE binding to ST of 83.7%. This reduction varied from 63.8% to 97.8%. Of the 20 patients, 7 of them showed an addition of the inhibitory effects of temperature and enzymatic digestion. In contrast, we noticed in 9 patients a higher decrease in their IgE binding of ST by pepsin effect than combined treatment.

Using dot-blot assay (Figure 6), the results showed that sera of 11 patients tested (with high IgE levels) detected the spots, indicating their reactivity towards native ST. The



Figure 4. Dot-blot assay of ST using rabbit IgG against native ST. 1, Dot-blot with native ST; 2, Dot-blot with ST treated by temperature; 3, Dot-blot with ST treated by pepsin; 4, Dot-blot with ST treated by the combination of two treatments.

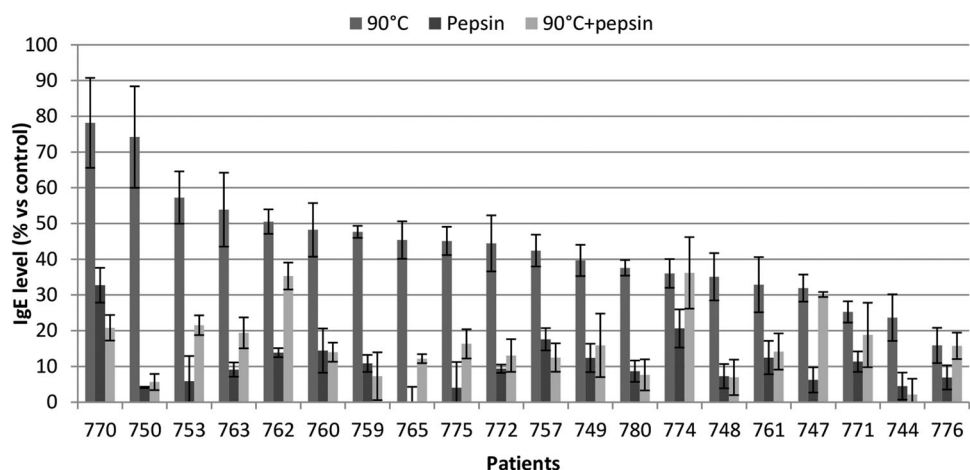


Figure 5. Effect of heating and enzymatic digestion on the recognition of ST by human IgE.

intensity of blotting spots decreased when ST was heated in 11 patients. Under digestion with pepsin, the IgE binding capacity was eliminated almost completely, as no spot was detected in all patients tested. When the two treatments were combined, 3 of 11 patients detected the spots, but with a weaker intensity than native ST profile.

Discussion

The objective of this study was first to evaluate the prevalence of self-reported fish/shellfish allergy and the IgE-sensitivity to ST in a Moroccan population from Fez region, and then

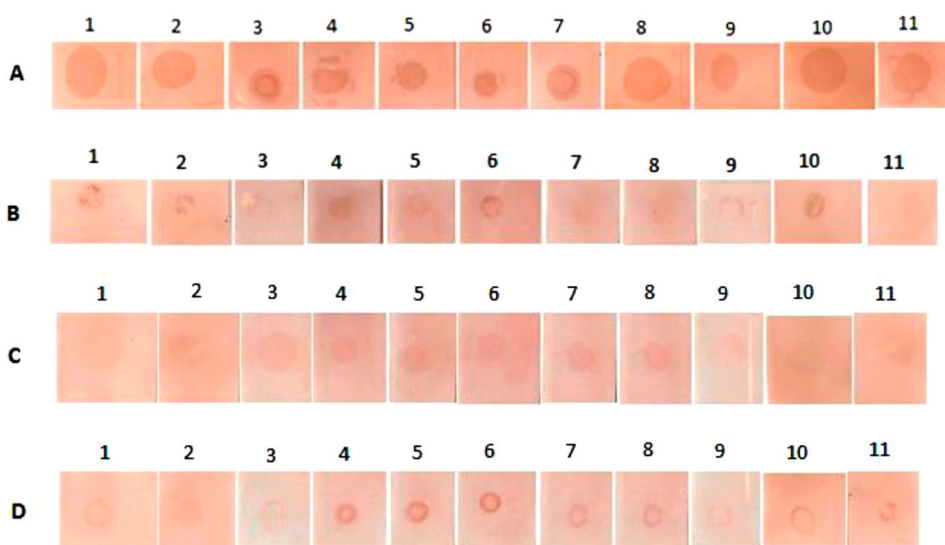


Figure 6. Results of Dot-blot assay with sera of 11 patients. (A) Dot-blot with native ST; (B) Dot-blot with ST treated by temperature; (C) Dot-blot with ST treated by pepsin; (D) Dot-blot with ST treated by the combination of two treatments.

to study the effect of temperature and enzymatic digestion on the allergenicity of ST. A total of 500 patients, aged between 1 and 60 years, from Fez Hospitals were surveyed using a questionnaire and their sera analyzed for specific IgE against ST.

The self-reported adverse reaction to fish and shellfish, evaluated in this population, was of 2.6% in children and increased to 12% in adults. In the world, several cases of fish/shellfish sensitizations have been reported in different countries. In children, the value observed, in this survey, was higher than that published in developed countries such as United States, Canada and Japan (Gupta et al., 2011; Noda, 2010; Soller et al., 2012). However, these reported values were seen to be increasing in emerging and less developed countries worldwide, such as Mexico, China, Philippines, Turkey, South Africa, Mozambique, Tunisia and Saudi Arabia (Aba-Alkhail & El-Gamal, 2000; ben Ameer et al., 2014; Gray & Kung, 2012; Kim et al., 2008; Lunet, Falcao, Sousa, Bay, & Barros, 2005; Orhan et al., 2009; Sánchez & Sánchez, 2015; Shek et al., 2010). This variation worldwide indicates that the value provided in this study on Fez region is close to values obtained in less developed countries, and is probably related to the quality of fish/shellfish products and their freshness related to histamine content. Thus, higher self-reported allergy prevalence in less developed countries could be explained by the effect of histamine content in those products which can induce false allergic reactions.

Concerning the IgE rates, we observed that adults (11.5%) had a high specific IgE to ST, compared with children (6%) population. These values were close to fish/shellfish reported sensitivity, but no correlation was observed in this population between fish/shellfish reported sensitivity and IgE rates. This no correlation was also observed in others studies between clinical tests and specific IgE levels (Čelakovská, Ettlerová, Ettler, Vaněčková, & Bukač, 2015; Lim et al., 2008; Perez-Gordo et al., 2013). Interestingly, Yang et al. (2010) found that measurement of IgE to ST by ELISA was superior to skin prick testing, suggesting that use of measurements of IgE to ST provided added value to the diagnosis of shrimp allergy.

The sera with high IgE level were selected and used for the evaluation of their reactivity towards ST treated. The different treatments include heating, pepsin, and a combination of pepsin and heat treatments.

Results showed that heating reduced slightly ST band profile, while we observed by ELISA a high reduction in the human IgE binding to ST, in all patients tested, with an average reduction of 56.7%. These results were confirmed by dot-blot assay, indicating that IgE binding reactivity was reduced in all patients tested. Several studies showed that high temperatures achieved during cooking break disulfide bridges of proteins, modifying their secondary and tertiary structures, and also their capacity to bind to IgE (Paschke & Besler, 2002; Poms & Anklam, 2004). Interestingly, Yu et al. (2011) observed that boiling accelerated tropomyosin digestion in simulated gastric fluid, which suggested that heating affects the structure and characterization of tropomyosin. Moreover, a recent study by Long, Yang, Wang, Hu, and Chen (2015), concerning the effect of heat treatment on the allergenic potential of ST, found a strong reduction with 73.59% in IgE binding to ST when the samples were treated at 55°C. Therefore, the results suggested that heating reduced IgE binding to ST and indicated that the antigenic epitopes of ST recognized by the human Moroccan IgE were almost conformational. However, remaining binding of heated ST to human IgE indicated that a part of epitopes involved were sequential.

After treatment with pepsin, SDS-PAGE profile indicated that the intensity of the original band ST was more decreased from that with heating treatments. This reduction was similar to results from previous studies on tropomyosin in digestion conditions by pepsin (Lin et al., 2015; Yu et al., 2011). Concerning IgE immunoreactivity to ST, results showed that pepsin hydrolysis altered the binding of human IgE with an average reduction of 89.4% for all patients tested. These results are consistent with dot-blot assay, which showed that the reactivity of IgE binding to hydrolyzed ST was almost eliminated in all patients tested. This indicates that the epitopes detected by human IgE were almost conformational. Similar studies demonstrated that protease digestion was effective in reducing the allergenicity of crustacean tropomyosin (Shimakura, Tonomura, Hamada, Nagashima, & Shiomi, 2005) and crab tropomyosin (Huang et al., 2010). Moreover, a recent study by Gámez et al. (2015) found that ST do not retain their IgE-binding capacity or their allergenic potency after digestion processing, which suggested that digestion treatment could be a good approach to reduce ST allergenicity. These data confirm our results which indicate the reduction of IgE binding on pepsin digested ST.

When the two treatments were combined (heating and pepsin), SDS-PAGE profile showed a higher decrease in intensity of the original band ST compared with other treatments. Furthermore, dot blotting and ELISA demonstrated that the reactivity of IgE binding of ST was reduced after treating for all patients tested. The average reduction in the IgE recognition of ST was 83.7%. Those results suggest that heating ST followed by pepsin hydrolysis did not alter more antibody epitopes. This indicates that the epitopes recognized by human IgE were almost conformational. However, some patients (3 of 20 patients) showed a slight increase in their IgE binding of ST by combined treatment compared to pepsin alone. This could be explained by the fact that the heating alone denatures conformational sites leading to decrease in IgE reactivity. The same way explained the pepsin action affecting these conformational epitopes. The combination of heating and pepsin may cause the generation of new epitopes, as a result of aggregation of pepsin-generated peptides during combination treatments leading to an increase in IgE reactivity (Mine & Yang, 2008).

In conclusion, these results showed that the Moroccan population reported sensitivity to ST confirmed by specific IgE measurement. Our study demonstrates that the processing procedure, such as heating and pepsin alone or in combination, produces a reduction on the SDS-PAGE protein profiles and IgE recognition of ST. These suggest that most epitopes recognized by Moroccans IgE were conformational and were altered by heating and enzymatic digestion.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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