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Sensitivity of Moroccans to sardine parvalbumin and effect of heating and enzymatic treatments

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ABSTRACT

The aim of this study was to evaluate the IgE sensitivity to sardine parvalbumin (SP) in a Moroccan population from Fez region, and then to study the effect of temperature and enzymatic digestion on the allergenicity of SP. This work was conducted with a questionnaire completed by a sera-bank, obtained from 1008 patients recruited from Fez Hospitals. Their sera were analyzed for specific IgE against SP. From the questionnaire, 8.1% reported adverse reactions to fish and shellfish, where sardine was the most common species causing adverse reactions in patients. Evaluation of specific IgE showed that 7.8% of patients present higher values. Further indirect ELISA and dot-blot results indicated that SP, a major fish allergen, showed a reduction in the human IgE binding under heating and pepsin hydrolysis. These results demonstrate that this population was sensitive to SP and the sensitivity could be reduced by heating and more where it was digested by pepsin.

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Sensitivity; sardine parvalbumin; human IgE; heating; pepsin hydrolysis

Introduction

Fish is one of the eight prominent foods known to cause allergy (Lopata & Lehrer, 2009). Fish is an important source of animal proteins for the Moroccan population. A large variety of fish are used for Moroccan consumption where Sardine (*Sardina pilchardus*) is the most widely consumed fish species. However, in Morocco, no study focused on fish sensitivity except Ghadi, Dutau, and Rance (2007), which showed that 4% of 160 atopic children in Marrakech city have sensitization to fish. In a neighboring country like Spain, fish was the third most common food sensitivity (after egg and cow's milk) (Pascual et al., 2008).

Among the various allergens characterized in fish, parvalbumin, a calcium-binding protein, has been recognized as the major allergen (Beale, Jeebhay, & Lopata, 2009; Bugajska-Schretter et al., 1998). It is a globular protein, about 12 kDa in size and is abundant in fish muscle (Girija & Rehbein, 1988). Research has demonstrated that more than 95% of fish-allergic patients have been found to have specific IgE to this protein and many

of the IgE-binding epitopes on this allergen were present in various fish species (Bugajska-Schretter et al., 1998; de Martino et al., 1990).

In order to reduce the risk of fish allergy, various food processing techniques described in the literatures such as heating, enzyme hydrolysis and pH can influence the allergenic potential of food proteins. These modifications may increase or decrease the allergenicity of proteins, depending on the severity of the treatment (Bousfiha & Aarab, 2013; Cabanillas et al., 2012; Ouahidi, Aarab, & Dutau, 2010; Ouahidi, El Hamsas, & Aarab, 2011; Thomas et al., 2007; Wang et al., 2014; Xu, Shi, Yao, Jiang, & Luo, 2016).

The aim of this study was first to evaluate the IgE sensitivity to sardine parvalbumin (SP) in a Moroccan population from Fez region, and then to study the effect of temperature and enzymatic digestion on the allergenicity of SP.

Material and methods

Patients

The study was conducted on a sera-bank, with data obtained from 1008 patients recruited in Fez city from the University Hospital Centre and from Ibn El Khatib –Hospital. It should be noted that they were chosen at random. They were people who came for different medical tests.

A questionnaire was provided to all patients concerning food allergy characteristics and fish allergy in particular. Patients were asked to provide information on the species they tried, the presence of allergic symptoms and types of fish allergy (sardine, mackerel, common Pandora, whiting, shrimps, etc.). This work was conducted from May 2014 to June 2015 and was approved by the Ethic committee of the University Hospital Center of Fez.

Serum collection

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

Extraction of SP

SP was extracted as previously described, with modifications (Hamada, Nagashima et al., 2003; Hamada, Tanaka et al., 2003). Briefly, the raw sardine muscle was defatted with chloroform to remove lipids. Once this preparation was filtered, the powder was dried overnight at room temperature. The samples were subsequently extracted with 10% of 0.15 mol/l NaCl in 0.01 mol/l phosphate buffer (pH 7.0), and boiled for 10 min. The heated extract was filtered and centrifuged at 3000 rpm for 15 min. Then, the resulting supernatants were dialyzed against distilled water overnight at 4°C . The dialyzed proteins were stored at -20°C until use for subsequent experiments. Extract quality was confirmed by means of sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE).

ELISA analysis

Specific IgE to SP was determined by indirect ELISA as previously described (Bousfiha & Aarab, 2013; Ouahidi, Aarab, & Dutau, 2010; Ouahidi, El Hamsas, & Aarab, 2011). Briefly, 100 µl of SP (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. Binding of anti-IgE was revealed by adding 100 µl of 0.05% orthophenylenediamine. The reaction was stopped by adding HCl 3M. Then the developed color was measured by absorbance at 490 nm.

SDS-PAGE and dot-blot assay

The extracted SP 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 SP was spotted on nitrocellulose membranes (5 µl for each dot) and left to dry at room temperature 37°C. The spotted membranes were saturated in 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 in BBS tampon. The intensity of spot indicates the reactivity of specific IgE to SP.

Effect of heating and enzymatic digestion

The purified SP (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, SP was digested with pepsin (30 µg/ml) in an acid environment (pH 2) for 30, 60 and 120 min at 37°C. Thereafter, the SP treated was deposited in 96-well microplates and human IgE binding evaluated by ELISA and dot-blot assay as described before.

Preparation of rabbit anti-parvalbumin antibodies

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

Statistical analysis

Descriptive statistics are 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

Sample description

A total of 1008 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 800 females (79.4%) and 208 males (20.6%). There were 79.6% patients, represented by adults, aged between 20 and 60 years, and 20.4%, represented by children, aged between 1 and 19 years (Table 1).

Reported adverse food reactions

In our study, we observed that the self-reported food adverse reactions, in these patients, were 10%. It was mostly associated with fish and shellfish (8.1%), followed by eggs (3.4%) and milk (1.9%). Regarding fish and shellfish, we observed that sardine (7.3%) was the most common species causing adverse reactions in patients, followed by horse mackerel (5.2%) and mackerel (4.4%). As regards age and sex, self-reported fish sensitivity was higher in adults (8.6%) than in children (6.3%) and higher in female (8.6%) than in male (6.3%). As regards the results of self-reported sardine sensitivity, we noted that the prevalence was most marked in adults (81%) than in children (19%) as well as in female (84%) than in male (16%). From this population, 97% reported of fried sardine sensitivity. The most frequent clinical signs were cutaneous reactions (77%), followed by gastrointestinal reactions (20%) and respiratory symptoms (8%).

Specific IgE measurement

Sera of 1008 patients have been tested for specific IgE binding to SP. The dosage of specific IgE to SP indicated that the average of positive values (>2 IU/ml) was 81.6 UI/ml (*n* = 657) varying between 2.75 and 359.42 UI/ml. From them, 19.2% of patients present values higher than 100 IU/ml and 7.8% concerning values higher than 150 IU/ml (Table 2).

Table 1. Demographic characteristics of the study population.

Demographic variable	Total samples		Self-reported fish adverse reactions	
	<i>N</i>	%	<i>N</i>	%
All	1008	–	82	8.1
Gender				
Female	800	79.4	69	8.6
Male	208	20.6	13	6.3
Age				
Children (<20 years)	205	20.4	13	6.3
Adults (>20 years)	803	79.6	69	8.6

Table 2. Distribution of IgE levels among the study population.

Dosage of specific IgE	>100 UI/ml	%	>150 UI/ml	%
Total	193	19.2	79	7.8
Gender				
Female	161	20.1	66	8.3
Male	32	15.4	13	6.3
Age				
Children (<20 years)	33	16.1	15	7.3
Adults (>20 years)	160	19.9	64	8

According to age and sex, the results showed a higher prevalence in female (8.3%) than in male (6.3%) and in adults (8%) than in children (7.3%) with a rate higher than 150 IU/ml.

SDS-PAGE of SP

The SDS-PAGE profiles of untreated SP and SP treated by temperature and pepsin are presented in [Figure 1](#). Parvalbumin proteins appeared as a band corresponding to the molecular mass 12 kDa, which is considered to be the major allergen in fish. Under temperature at 90°C for 1 h, SDS-PAGE profile indicated a small decrease in the intensity of SP band after heating (lane 2) compared to the original band (lane 1). Concerning the effect of pepsin hydrolysis, there was no clear protein band was appeared in lane 3, which the intensity of SP band became almost disappeared. By contrast, when SP was exposed to both treatments (heating followed by pepsin hydrolysis), we noted a SP band in lane 4 but with a weaker intensity than that with heating effect.

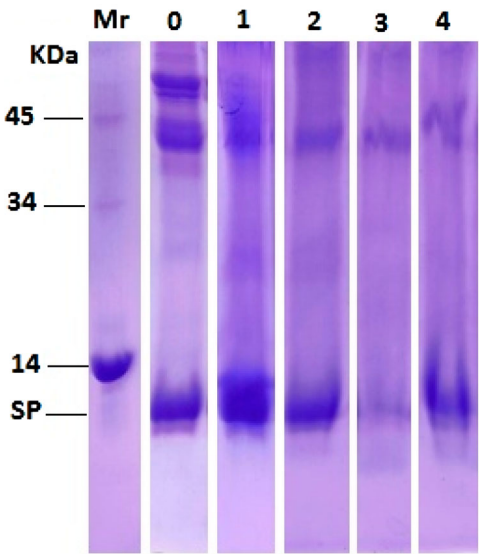


Figure 1. SDS-PAGE analysis of untreated SP and SP treated by heating and pepsin.

Notes: Mr, markers of the molecular weights; 0, total proteins of sardine extracted in phosphate buffer solution (10%, pH 7.4); 1, extracted native SP; 2, SP treated by temperature; 3, SP treated by pepsin and 4, SP treated by the combination of two treatments.

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

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

Results (Figure 2) showed that the IgG binding to SP was slightly decreased at 80°C after 30 min of treatment and was highly diminished at 90°C. The maximum reduction observed was 65.3%. Concerning the effect of enzymatic treatment (Figure 3), we noticed that SP pepsin hydrolysis altered the binding of IgG to SP. Furthermore, pepsin hydrolysis combined with thermal treatment at 90°C inhibited the IgG binding by the same effect with pepsin alone. The maximum reduction observed was 67% with pepsin alone and 66% with combination of two treatments.

This decrease in reactivity was confirmed by dot-blot analysis (Figure 4). The dot-blot assay achieved by the anti-SP rabbit IgG showed the presence of a spot corresponding to parvalbumin, indicating the reactivity of rabbit IgG toward SP. After treatments, we noted that heating at 90°C reduced the intensity of blotting spot, while pepsin hydrolysis (alone or in combination with heating) removed this reactivity.

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

Human sera were analyzed for their IgE sensitivity to SP. Human sera of 20 patients with higher IgE levels (>100 IU/ml) were selected and used to estimate the binding variation of IgE to SP under temperature at 90°C and enzymatic treatment (during one hour) using ELISA (Figure 5) and dot-blot assays (Figure 6).

Under heat treatment (Figure 5), we observed that 18 of 20 patients showed a decrease in the IgE binding to protein SP from 4.7% to 54.5% with an average diminution of 27.7%. In contrast, we noticed a small increase of recognition under this treatment in two patients. Where SP was treated by pepsin, we observed for all patients a reduction in IgE recognition varying from 34.9% to 76.9% with an average of 57.9%. When the two treatments were combined (heating 90°C followed by pepsin), we observed an average

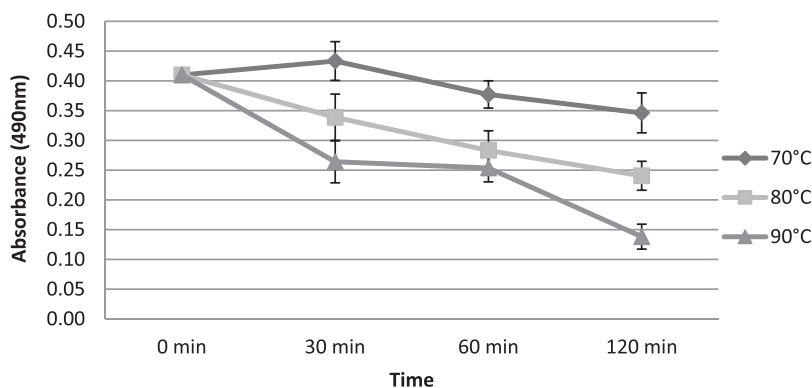


Figure 2. Effect of different temperatures on the recognition of SP by rabbit IgG.

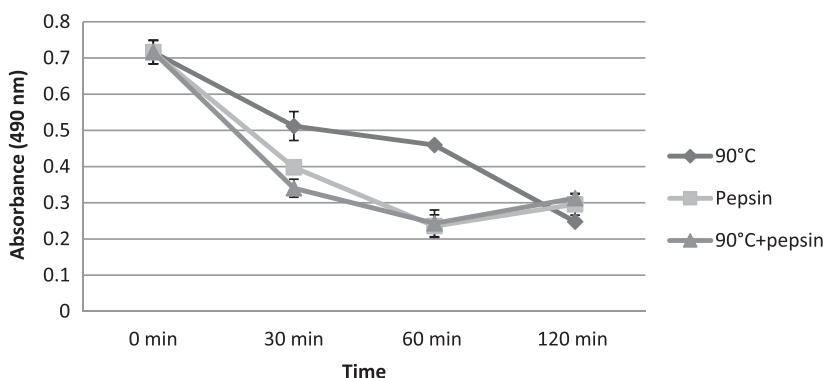


Figure 3. Effect of heating and enzymatic digestion on the recognition of SP by rabbit IgG.

diminution in the IgE binding to SP of 46.6%. This reduction varied from 22.9% to 75.6%. Of these patients, three of them showed an addition of the inhibitory effects of temperature and enzymatic digestion. In contrast, we noticed in the rest a higher decrease in their IgE-binding of SP by pepsin effect than combined treatment.

In the blotting assays (Figure 6), the sera from 10 patients with high IgE levels were used to recognize SP. Results showed that all the sera tested detected the spots, indicating their reactivity toward native SP. The intensity of blotting spots decreased in all patients when SP was heated at 90°C. Under digestion with pepsin, the IgE-binding capacity was eliminated completely, as no spot was detected in all patients tested. The same result was observed when the two treatments were combined, whereas a spot was detected in one patient, but with a weaker intensity than native SP profile.

Discussion

The aim of this study was first to evaluate the IgE sensitivity to SP in a Moroccan population from Fez region, and then to study the effect of temperature and enzymatic digestion on the allergenicity of SP. This work was conducted with a questionnaire completed by a sera-bank, obtained from 1008 patients recruited from Fez Hospitals. These patients were questioned and their sera were analyzed for specific IgE against SP.

The self-reported adverse reaction to fish and shellfish, evaluated in this population, was of 6.3% in children and increased to 8.6% in adults. In children, the value observed 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



Figure 4. Dot-blot assay of SP using rabbit IgG against native SP.

Notes: 1, Dot-blot with native SP; 2, dot-blot with SP treated by temperature; 3, dot-blot with SP treated by pepsin and 4, dot-blot with SP treated by the combination of two treatments.

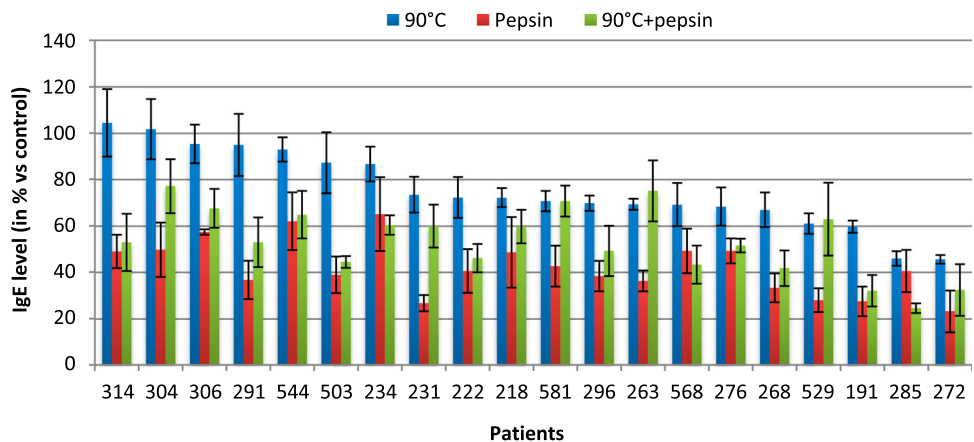


Figure 5. Effect of heating and enzymatic digestion on the recognition of SP by human IgE.

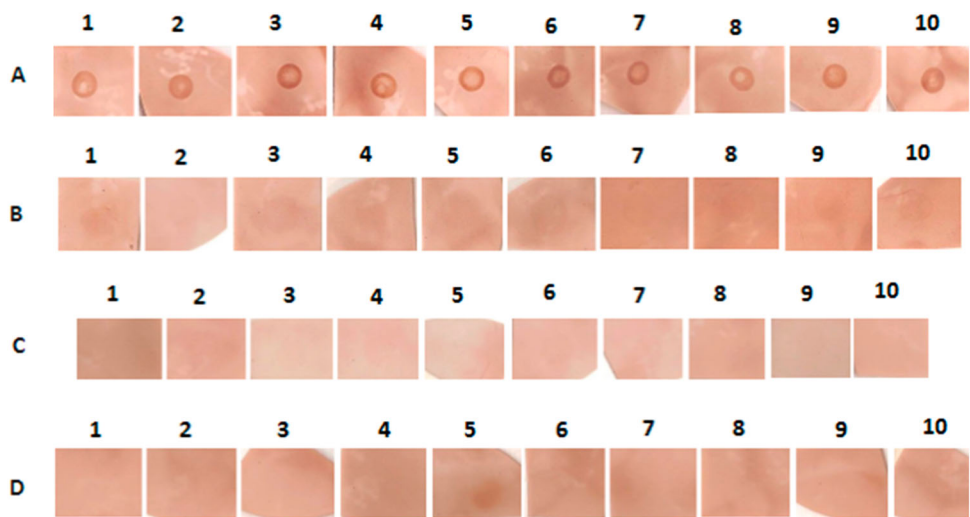


Figure 6. Results of dot-blot assay with sera of 10 patients. (A) Dot-blot with native SP, (B) dot-blot with SP treated by temperature; (C) dot-blot with SP treated by pepsin and (D) dot-blot with SP treated by the combination of two treatments.

values were seen to be increasing in emerging and less developed countries worldwide, such as Tunisia, Saudi Arabia, South Africa, Mozambique and Turkey (Aba-Alkhalil & El Gamal, 2000; ben Ameer et al., 2014; Gray & Kung, 2012; Lunet, Falcao, Sousa, Bay, & Barros, 2005; Orhan et al., 2009). 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.

Regarding the IgE rates, we observed that 7.3% of children and 8% of adults had a high specific IgE to SP. 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 other studies between clinical tests and specific IgE levels (Čelakovská, Ettlerová, Ettler, Vaněčková, & Bukač, 2015; Lim et al., 2008; Perez-Gordo et al., 2013).

The sera with high IgE level were selected and used for the evaluation of their reactivity toward SP treated by heating and/or pepsin hydrolysis. Dot-blot and indirect ELISA were used to analyze the human IgE binding of SP. The results showed that heating caused a small decrease in SP profile compared to native SP. Further ELISA noticed that the human IgE binding to SP was decreased after heating at 90°C with an average diminution of 27.7%. These results were confirmed by dot-blot assay, indicating that IgE-binding reactivity was reduced in all patients tested. Several studies have shown that heat processing has a profound effect on antibody reactivity to fish parvalbumins. Shibahara, Uesaka, Wang, Yamada, and Shiomi (2013) found that the reactivity on pacific Mackerel parvalbumin was considerably reduced at 121°C and only 18% of the original reactivity remained after heating for 60 min. In similar study, Cai et al. (2010) showed that the IgE-binding activity to red stingray parvalbumin was decreased gradually with an increase in temperature greater than 80°C. Moreover, Saptarshi, Sharp, Kamath, and Lopata (2014) showed that heat processing caused a reduction in antibody reactivity to multimeric forms of parvalbumins for most bony and cartilaginous fish. These works indicate that heating is responsible for reduction of IgE binding to SP. In fact, the sensitivity of human IgE to SP in our Moroccan population was less reduced by heating than in Asian and Australian population. This reduction by temperature indicates the thermolability of some parvalbumin epitopes recognized by human IgE, and suggests that these epitopes are almost conformational. However, remaining binding of heated parvalbumin to human IgE indicated that a large part of epitopes involved were sequential.

After treatment with pepsin, SDS-PAGE profile showed a higher decrease in intensity of the original band SP compared with heating. Concerning IgE immunoreactivity to SP, we noticed that pepsin hydrolysis altered the binding of IgE to SP for all patients tested, with an average reduction of 57.9%. The same result was observed with dot-blot assay, which showed that the reactivity of IgE binding to hydrolyzed SP was eliminated completely in all patients tested. Generally, protease digestion is known as an effective way to reduce the allergenicity of foods (Bousfiha & Aarab, 2013; Cai et al., 2010; Kobayashi, Hashimoto, Taniuchi, & Tanabe, 2004; Shimakura, Tonomura, Hamada, Nagashima, & Shiomi, 2005; Untersmayr et al., 2005). Several studies have shown a decrease in the allergenicity of parvalbumin by digestion processing. Untersmayr et al. (2003) noticed that caviar parvalbumin was completely degraded by pepsin, resulting in the loss of reactivity with IgE antibodies. This result was reached by De Jongh et al. (2013) which showed that the IgE binding to codfish parvalbumin was strongly diminished by enzymatic digestion processing. These data confirm our results which indicate the reduction of IgE binding on pepsin digested SP.

When the two treatments were combined (heating and pepsin), the intensity of the original band SP was highly decreased, and the IgE recognition of SP was reduced too for all patients, with an average diminution of 46.6%. The same decrease was obtained with dot-bolt assay, which results showed that the intensity of spots was almost

disappeared in all patients. Those results suggest that heating parvalbumin denaturation followed by pepsin hydrolysis did not alter more antibody epitopes. This indicates that the epitopes recognized by our Moroccan population studied were almost conformational.

In conclusion, these results demonstrate that the Moroccan population-reported sensitivity to SP is confirmed by specific IgE measurement. Our study has shown that SP, a major allergen, showed a reduction in its immunoreactivity with human IgE under heating and more where it was digested by pepsin. 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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