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The effect of treatments on the allergenicity of β -lactoglobulin in Moroccan population

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Treatments
on the
allergenicity

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Abstract

Purpose – This study aims to evaluate the effect of heating and pepsin hydrolysis on the allergenicity of purified cow's milk β -lactoglobulin using individual sera from Moroccan population.

Design/methodology/approach – A cross-sectional study was carried out in Hospitals of Fez-Meknes region, to evaluate specific IgE to raw cow milk, as well as to heated and pepsin-hydrolyzed β -lactoglobulin.

Findings – Results showed that 6.6 per cent of studied patients presented high values of serial IgE. The effect of treatments on the allergenicity of β -lactoglobulin showed that heating at 90°C and pepsin hydrolysis, for 1 h each, showed an important decrease in the recognition of human IgE with a maximum of reduction of 81 and 91 per cent, respectively.

Originality/value – This reduction of immunoreactivity of human IgE to treated β -lactoglobulin suggested that this Moroccan population recognized mostly conformational epitopes.

Keywords Heating, Cow's milk allergy, Human IgE, Pepsin hydrolysis, β -lactoglobulin

Paper type Research paper

Introduction

Cow milk contains about 3.5 per cent of proteins including caseins and whey proteins known to be most allergenic molecules. Cow's milk allergy, which is an immunologically mediated reaction against milk proteins, is one of the most common causes of food allergy in the world. Epidemiological studies have reported that cow's milk protein allergy was the most prevalent allergy for infants or young children, with an incidence of about 2 to 7.5 per cent in population-based studies in different countries and may also persist in adult patients (Fiocchi *et al.*, 2010; Burks *et al.*, 2001). In Morocco, cow's milk allergy is about 6.9 per cent in schoolchildren (Azzad *et al.*, 2016) and between 2 to 3 per cent in general population (Ouahidi *et al.*, 2017; Mejrhit *et al.*, 2017b). For approximately 60 per cent of cow's milk, adverse reactions are because of IgE-mediated allergy. Though, for patients without cow's milk-specific IgE, the mechanisms are not yet understood (Niggemann, 2002).

The main cow's milk allergens are caseins, β -lactoglobulin (β -LG) and α -lactalbumin (Bu *et al.*, 2013). The Whey proteins fraction is composed of α -lactalbumin, β -LG, immunoglobulins, bovine serum albumin and traces of lactoferrin. It has been found that the human IgE response to cow's milk proteins is characterized by a great variability, and sensitivities to various cow's milk proteins are widely distributed and not limited to a single protein (Wal, 2002; Hochwallner *et al.*, 2010; Alessandri *et al.*, 2012).

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β -LG, which is the major whey protein of most mammalian species, consisting of a small protein of 18.3 kDa and 162 amino acids in a dimeric form (Adams *et al.*, 2006; Niemi *et al.*, 2008). It has been indicated that β -LG presented a remarkable allergenic activity in addition to its absence in human milk (Wal *et al.*, 1995; Wal, 2002, 2004; Järvinen *et al.*, 2001).

It is frequently mentioned in the literature that several food processing techniques such as heating, enzyme hydrolysis and pH can influence the allergenic potential of β -LG. Heating, the most common industrial process of milk, induces significant changes in protein structure which may influence the IgE binding and allergenicity (Kosti *et al.*, 2013). Also, proteins can be broken down by enzyme hydrolysis into small peptide molecules and amino acids which may lead to reduce their allergenicity. However, different investigations on the effect of food processing on the allergenicity/antigenicity of cow milk proteins showed discrepancies. In fact, treatments may increase, decrease or even let unchangeable the allergenicity of milk proteins (Ehn *et al.*, 2004; Ehn *et al.*, 2005; Puerta *et al.*, 2006; Peyron *et al.*, 2006; Bu *et al.*, 2009; Duan *et al.*, 2012; Bøgh *et al.*, 2013; Verhoeckx *et al.*, 2015). In our previous research studies, we have observed that the IgE-binding levels of heated casein and α -lactalbumin decreased for the majority of studied patients but more significantly for α -lactalbumin than casein (Azdad *et al.*, 2017, 2018).

The aim of the present study is to evaluate the effect of treatments (heating and enzymatic hydrolysis) on the allergenicity of the purified cow's milk β -LG using individual sera of Moroccan population basing on ELISA and Dot-blot assay.

Material and methods

Patients

A transversal study was conducted in two hospitals of Fez-Meknes region, Hospital Ibn Elkhatib and the University Hospital Centre of Fez, and in private centers of medical analysis from September 2014 to February 2016. The distribution and collection of information on food allergy was done by contact direct with all participants. All collected questionnaires were verified according to our inclusion criteria. The principal inclusion criteria were the absence of any complicated health problems of the participants and the absence of any beforehand challenge with milk. The questionnaire contains questions about personal data, including gender, age and if there is any food causing them to experience hypersensitivity. If any current adverse reaction to cow's milk was reported, they were asked to describe the symptoms and if they had taken any medications. The symptoms were classified to skin reactions, gastrointestinal and respiratory symptoms. Then, blood samples were collected, centrifuged at 3000 rpm/5 min and stored at -20°C until use. Before any serum sample taking, a formal consent of each patient or of the children's parents was signed. This study was approved by the ethic committee of the University Hospital Center of Fez.

Extraction of β -lactoglobulin

The extraction of β -LG was taken in according to Wal *et al.* (1995). Briefly, a volume of 100 ml of cow's milk was skimmed, its pH was adjusted to 4.6 by HCl (3 mol/L) and centrifuged at 5000 rpm/20 min. The whey proteins were extracted in the supernatant dialyzed against bi-distilled water. The dialyzed extract was separated on G-100 Sephadex column and the absorbance of fractions (2 ml per tube) was determinate at 280 by an UV-Visible Spectrometer. The fraction presenting β -LG was then concentrated in a 10 per cent

polyethylene glycol solution (PEG). The quality of proteins extracted was characterized by SDS-PAGE.

SDS-PAGE and dot-blot assay

SDS-PAGE was executed in 20 per cent polyacrylamide gel beneath denaturation conditions. In brief, the loading buffer (10 per cent SDS, 10 per cent glycerol, 10 per cent β -mercaptoethanol and 2.5 per cent bromophenol blue) was mixed with β -LG (100 μ l) and heated at 100°C for 5 min. The migration was completed in electrophoresis chamber under a 25-mA current. Finally, the gel was stained with 0.1 per cent Coomassie blue R250.

Dot-blot assay was realized as described previously (Mejrhit *et al.*, 2017a, 2017b; Azdad *et al.*, 2018). The purified β -LG (5 μ l), native and treated were spotted on nitrocellulose membranes and incubated at 37°C for 2 h. Thereafter, to block the nonspecific binding sites, the dried spotted membranes were saturated by BBS-Tween (2.5 per cent) for 1 h at 37°C. The nitrocellulose membranes were then incubated with human sera at 4°C overnight and with anti-IgE peroxidase conjugate for 2 h at 37°C. Finally, the spots were revealed by the incubation of membranes in a solution of 0.05 per cent Diaminobenzidine (DAB) in BBS tampon. Each step of the analysis was followed by washing by BBS-Tween (0.1 per cent) two to three times.

Heat treatment and pepsin hydrolysis

β -LG underwent three different treatments: the first one was heating where the protein was treated by temperatures of 70, 80, and 90°C for 30, 60, and 120 min, respectively. The second one was pepsin hydrolysis where the protein was digested by pepsin (hog stomach, 3354 U/mg) at a concentration of 50 μ g/ml in an acidic medium (pH = 2) during 30, 60 and 120 min at 37°C. The third one was the combination of the two treatments where the protein is heated and then hydrolyzed by pepsin.

Production of polyclonal anti β -lactoglobulin antibodies

The preparation of anti β -LG antibodies was done by immunization of rabbits against the native β -LG using Freund adjuvant. After four weeks, animals were sacrificed according to National Ethical Laws and blood was collected in dry tubes. The collected blood was centrifuged for 15 min at 3,000 rpm at 4°C, and the serum was separated and then frozen at -20°C until use.

Specific IgE measurement

The measurement of specific IgE levels to raw milk and to β -LG was executed by indirect ELISA as described previously (Ouahidi *et al.*, 2011; Bousfiha and Aarab, 2013; Azdad *et al.*, 2017). First, 100 μ l of skimmed raw milk or β -LG at a concentration of 0.5 mg/ml in PBS (Phosphate Buffered Saline, pH 7.4) was deposited on the wells of micro-titration plate (100 μ l/well). Next, wells were saturated by borate buffered saline (BBS, pH 8.4) containing 2.5 per cent Tween 20 and 100 μ l of the human serum added. The revelation was made by adding the anti-human IgE conjugated to peroxidase followed by addition of the Orthophenylenediamine (OPD 0.05 per cent) substrate. After incubation at 37°C during 20 min, reaction was stopped, by adding 50 μ L of HCl (3 mol/l) and the absorbance was measured at 490 nm by an ELISA reader.

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 $p < 0.05$ as the limit of the significant value. The results are expressed as mean $\pm$ SEM (standard errors of mean), and the SEM was calculated by the following relation: $SEM = SD \text{ (standard deviation)}/\sqrt{n}$ (sample size). All statistical analyses were performed using Excel logical.

Results*Reported allergy*

Our studied population was constituted of 832 people; 45.4 per cent ($n = 454$) were women and 54.5 per cent ($n = 378$) were men. The age of the studied population ranged from 2 to 60 years old with 63.2 per cent aged from 20 to 40 years. Food allergy was reported by 18.1 per cent ($n = 151$) of the studied population where milk allergy was at 3.6 per cent. It was mostly caused by cow milk, 96.6 per cent ($n = 29$). As regards to dairy product, pasteurized milk, milk beverage and cheese were the most reported as causing adverse reactions with 36.6, 33.3 and 30 per cent, respectively. Most reported symptoms were gastrointestinal reactions by 71 per cent, and cutaneous reactions by 18 per cent of persons reporting milk allergy.

Specific IgE to raw cow milk

Specific IgE to cow's raw milk was realized for 557 of benevolent patients from the studied population. The results showed that 54.4 per cent ($n = 303$) presented positive values (>2 IU/ml) of serial IgE level ranging from 2.7 IU/ml to 595 IU/ml, with an average of 95.2 IU/ml, as well as 17.2 per cent ($n = 96$) presented values more than 100 IU/ml and 6.6 per cent ($n = 37$) more than 200 IU/ml.

Regarding age, 4.3 per cent ($n = 6$) of children and 9.1 per cent ($n = 29$) of adults presented values more than 200 IU/ml with an average of positive values of 85.3 IU/ml and 200 IU/ml, respectively. While, basing on sex factor, the average of positive values of IgE levels was 97.9 IU/ml for males and 94.8 IU/ml for females. Whereas, IgE level values more than 200 IU/ml were presented by 7.3 per cent of females and 3.9 per cent of males.

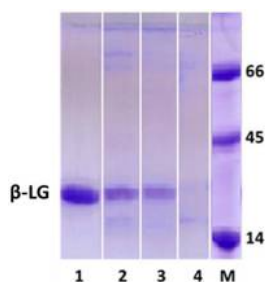
From the population presenting high levels of IgE to raw cow milk (up to 100 IU/ml), we screened 90 sera samples to determinate their specific IgE to β -LG. The results showed that 35 per cent (32/90) of the analyzed sera presented positive values to β -LG with an average of 70.5 IU/ml. These sera were then used to study the effect of treatments on the allergenicity of β -LG.

Electrophoresis of β -lactoglobulin

The solutions of purified β -LG native and treated by different treatments were showed in [Figure 1](#). The native β -LG appeared as a band corresponding to the molecular weight of 18 kDa. The treatment of this protein by heat showed a slight reduction in its band, but this later was totally disappeared when the treatment was either by pepsin or by the combination of the two treatments.

Effect of treatments on the antigenicity of β -lactoglobulin

In the aim to analyze the effect of heating and pepsin hydrolysis on allergenicity of β -LG, three sets of experiments were conducted. We have studied the action of heating, pepsin digestion and the two treatments combined. The detection of treated β -LG was evaluated by rabbit IgG anti- β -LG basing on ELISA and Dot-blot assay.



Notes: Native β -LG (1); β -LG treated by temperature (2); β -LG hydrolyzed by pepsin (3); β -LG treated by combination of the two treatments (4). Molecular weights markers (M); The band of β -LG is located in the position of 18 KDa. The band intensity decreased after heat and enzymatic treatments. The band disappeared after undergoing the combination treatment

Treatments
on the
allergenicity

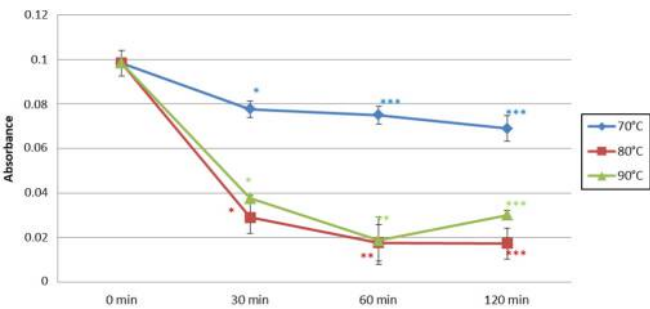
Figure 1.
Electrophoresis
profile of β -LG

According to Figure 2, the detection of β -LG was reduced after heating within 30 min at different temperatures and was slightly modified for more heating time. Temperature of 80°C and 90°C highly changed the liaison to IgG more than 70°C. Maximal reduction of IgG binding for β -LG was 30 per cent at 70°C, 82 per cent at 80°C and 82 per cent at 90°C.

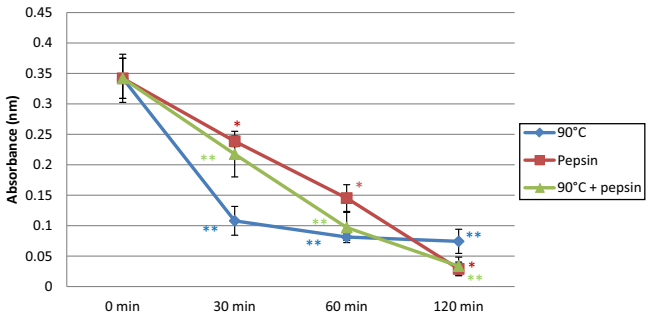
The result of enzymatic treatment showed that pepsin hydrolysis of β -LG almost completely blocked the detection by rabbit IgG after 2 h (Figure 3). Also, after undergoing a combination of treatments, the effect of reduction was faster allowing to a reduction of more than 70 per cent of the immunoreactivity just after 1 h of treatment with a high reduction after 2 h (Figure 2). This result was confirmed by the Dot-blot assay where the reactivity of β -LG to IgG was totally attenuated when the two treatments were combined (Figure 4).

Effect of treatments on the allergenicity of β -lactoglobulin

The results of the study of the variation of β -LG allergenicity using human IgE were presented in Figure 5. Concerning the reaction to heated β -LG at 90°C about 60 min, all studied patient showed an interesting decrease in the recognition of the treated protein with a maximum of reduction of 81 per cent and an average of 53 per cent. Under pepsin hydrolysis, the results showed that all of the patients presented a high reduction in the recognition of treated β -LG. This reduction was varying from 8 to 91 per cent and it was more than 40 for 75 per cent of studied patients ($n = 15$ from 20). In the same manner, high



Notes: Student's *t*-test, any value of $p < 0.05$ was considered statistically significant. * $p < 0.01$; ** $p < 0.1$; *** $p = 0.2$ compared to control (native β -LG) at $t = 0$ min



Notes: Student's *t*-test, any value of $p < 0.05$ was considered statistically significant. * $p < 0.01$; ** $p = 0.05$; compared to control (native β -LG) at $t = 0$ min

Figure 2.
Effect of heating on β -LG binding to rabbit IgG

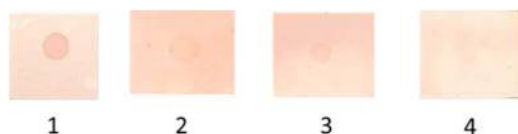
Figure 3.
Effect of heating and pepsin hydrolysis on β -LG binding to rabbit IgG

decrease reactivity to treated β -LG by combination of heating and pepsin hydrolysis was observed for these studied patients with a reduction average of 50 per cent and a maximum of reduction of 91 per cent. However, we noticed that one patient showed an increase in the recognition of β -LG after the combination of treatments.

Basing on Dot-blot assay, sera of 12 patients (with high IgE levels) were tested on native β -LG. The presence of spots indicated that all these patients reacted to native β -LG. Thus, their sera were used to study their reactivity to the treated protein. The results showed that the intensity of the spots has significantly decreased when the protein was treated by heat for all studied patients. As regards to the pepsin hydrolysis and the combination of the two treatments, we remarked that they were more efficient in reduction of the spots or their disappearance indicating that the reactivity of IgE of these patients to treated β -LG was strongly attenuated (Figure 6).

Discussion

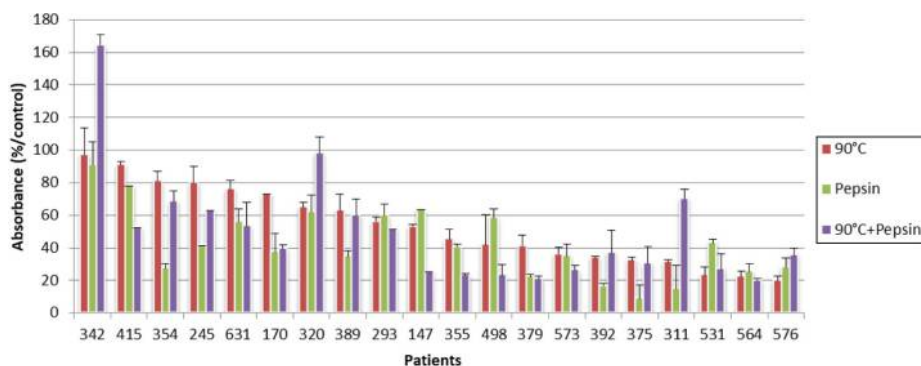
The objective of this research was to study in Moroccan population, the variation of specific IgE immunoreactivity to treated cow's milk β -LG, using ELISA and Dot-blot assay.



Notes: Dot-blot with native β -LG (1); Dot-blot with β -LG treated by temperature (2); Dot-blot with β -LG treated by pepsin (3); Dot-blot with β -LG treated by combination of the two treatments (4); The reactivity of rabbit IgG to native β -LG showed an intense spot. This spot intensity decreased slightly after heat treatment (2) and then strongly after pepsin hydrolysis (3) as well as after the combination of treatments (4)

Treatments
on the
allergenicity

Figure 4:
Dot-blot assay of
 β -LG using rabbit
IgG anti β -LG

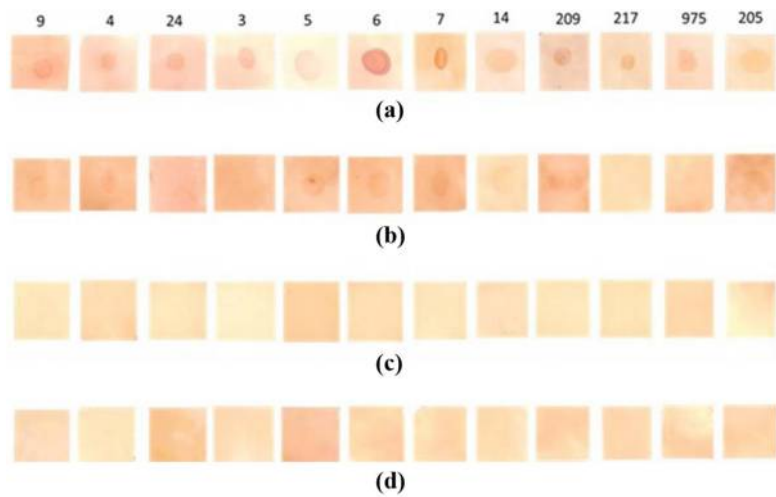


Notes: The percentage of reduction was calculated according to native β -LG and presented as mean (%) $\pm$ Standard Error. All studied patients ($n = 20$) showed a decrease in their IgE levels to treated β -LG

Figure 5.
Effect of heating,
pepsin hydrolysis
and combination of
the two treatments on
the detection of β -LG
by human IgE

This Moroccan population reported Milk allergy by 3.6 per cent. Basing on determination of IgE levels to cow milk proteins, we found that 17.2 per cent of the studied population presented high values to raw milk. From whom, 6.8 and 1.8 per cent presented high levels of specific IgE to casein and to β -LG, respectively. This later indicated a less sensitivity of our Moroccan population to milk β -LG compared to casein.

The evaluation of the effect of heat and enzymatic treatments on β -LG recognition by human IgE showed that there was a significant decrease in the β -LG-specific IgE levels for all patients. The majority of the studied patients (80 per cent) presented a predominant decrease in the recognition of heated β -LG and this decrease reached 89 per cent of reduction which starts and persists all over the first hour of treatment. This result is in accordance with previous findings (Taheri-Kafrani *et al.*, 2009; Morisawa *et al.*, 2009; Baldo, 1984) using individual sera of allergic persons, as well as with pooled sera (Ehn *et al.*, 2004). Likewise, authors find same conclusion using whole milk (Bloom *et al.*, 2009; Nowak-Wegrzyn *et al.*, 2008; Norgaard *et al.*, 1996). However, Høst and Samuelsson (1988) found that pasteurized milk evoked allergic reactions in children allergic to milk. From our population, 20 per cent of patients presented a



Notes: (a) Dot-blot with native β -LG; (b) Dot-blot with β -LG treated by temperature; (c) Dot-blot with β -LG treated by pepsin; (d) Dot-blot with β -LG treated by combination of the two treatments; The presence of spots indicated that all the studied patients ($n = 12$) presented specific IgE to native β -lactoglobulin (A). The intensity of the spots decreased after heat treatment (B). These spots disappeared when β -lactoglobulin underwent enzymatic hydrolysis as well as when enzymatic hydrolysis was preceded by heat treatment (C and D, respectively)

Figure 6.
Results of dot-blot
assay with sera of 12
patients

slight decrease in the recognition of the heated protein. This finding could be explained by the characteristics of the immunologic response of the studied population which indicates that the majority of patients recognized conformational epitopes more than linear ones.

The study of IgE immunoreactivity to hydrolyzed β -LG showed that pepsin hydrolysis attenuated its recognition by human IgE in all studied patients. This reduction was presented by an average of 42 per cent for all the studied persons and attended more than 70 per cent for one third of them. This result obtained with Moroccan patients is consistent with data published by Asselin *et al.* (1989), Selo *et al.* (1999) and Ehn *et al.* (2005) regarding populations of Canada, Sweden and France. Selo *et al.* (1999) found that some patients allergic to β -LG presented high specific IgE levels to peptides generated from tryptic digestion of β -LG more than the native β -LG. Recently, Charcosset *et al.* (2016) found that the IgE binding of France milk allergic patients, especially to β -LG, was not reduced after pepsin digestion of β -LG, while it was significantly reduced after hydrolysis using α -chymotrypsin. Hence, the immunological characteristics of our population seem to be similar to some populations but not to others, confirming the idea that genetic make-up of the individual and their sensitivity against the allergen could influence the effect of enzymatic hydrolysis on the potential allergenic of proteins.

When β -LG underwent heating followed by pepsin hydrolysis, 95 per cent of the studied patients presented significant decrease in the recognition of the treated protein. Also, almost half of this Moroccan population showed an important decrease in the recognition of the treated β -LG which was higher than heat and hydrolysis treatment each one solely as observed before (Bousfiha and Aarab, 2013; Ouahidi *et al.* 2017; Mejrihit *et al.* 2017a). By using pooled sera of French patients with cow's milk sensitivity, Peyron *et al.* (2006) observed that previous heating of

β -LG induced a high increase of peptic digestion leading to a reduction of the antigenicity and allergenicity of the treated β -LG. However, in our case, three patients presented an increase in the IgE level after the combination of heating and hydrolysis. This response might be explained by either the creation of new epitopes or by exposition of masked linear epitopes because of successive treatments as suggested before (Monaci *et al.*, 2006; Mejrhith *et al.*, 2017b; Ehn *et al.*, 2005). The reduction of human IgE reactivity might be explained by the fact that our Moroccan population recognizes mostly conformational epitopes which are degraded under pepsin hydrolysis, as well as under the combination of treatments, as it is indicated by SDS-PAGE.

Conclusion

To sum up, our findings showed that this Moroccan studied population was less sensitive to milk β -LG than casein. The treatment of β -LG revealed an important decrease in its binding to human IgE after heat treatment, pepsin hydrolysis and after their combination. This result exhibits that the studied population recognizes mostly conformational epitopes and these treatments could be used to reduce the allergenicity of the major allergen of cow's milk whey proteins. Knowledge of the epitopes predilection of our population can assist in looking for new processing methods that may lead to the elimination of residual allergic epitopes, as well as to make correct choice of the hypoallergenic products that should be consumed. Also, these results will be of interest to the medical staff more particularly clinicians working with allergic persons to take in consideration that they should perform clinical tests, such as skin prick test, with treated milk next to raw milk, since we found that some patients still react to treated β -LG.

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