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Reduction of the allergenicity of cow's milk α-lactalbumin under heat-treatment and enzymatic hydrolysis in Moroccan population

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Key words

serial IgE; α-lactalbumin; heat-treatment; pepsin hydrolysis; epitopes

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Summary

The aim of the present study is to evaluate the effect of heat-treatment and enzymatic hydrolysis on the allergenicity of cow's milk α-lactalbumin (α-LA) in a Moroccan population. A total of 557 patients were recruited from the University Hospital Complex and the Ibn El Khatib Hospital of Fez city. This population consented to realize a dosage of IgE levels to raw cow milk and then to α-LA native and treated with the studied treatments. The results revealed that 54.4% of the studied subjects presented positive values of serial IgE to raw cow milk. The effect of treatments on the allergenicity of α-LA showed that heat-treatment at 90°C and pepsin hydrolysis at 37°C, for 1 hour each, caused an important decrease in the IgE binding with an average of reduction of 59% and 74%, respectively.

Introduction

Cow's milk is the first component introduced into the diet, and it is the most common cause of food allergy in the World. In Morocco, cow's milk allergy is about 6.9% in schoolchildren (1) and between 2% to 3.6% in general population (2,3,4).

Several studies have identified casein as a major cow milk allergen that induces strong immediate allergic reactions (5,6,7). β-Lactoglobulin represents another important cow milk allergen that is recognized by milk allergic patients (8,9). However, for α-lactalbumin, a widely varying sensitivity has been reported in the literature (10,5).

The α-lactalbumin is a 14.2 kDa calcium binding protein, which plays an important role in the biosynthesis of lactose through the interaction with lactose synthase (11). It is expressed exclusively during lactation in the mammary gland and accounts for 20% of bovine whey proteins (12).

Different studies have been reported concerning the effect of treatments on the allergenicity of cow's milk proteins, indicating either a decrease or an increase in the sensitivity of patients (13,14,15,16,4). However, studies about the modification of allergenicity of α-lactalbumin were limited.

From the above, the purpose of this research is to determine the effect of thermal treatment and enzymatic hydrolysis on the antigenicity as well as the allergenicity of α-lactalbumin in a population from Fez-Meknes region of Morocco, using ELISA and Dot-blot assay.

Materials and methods

Collect of patient's sera

A transversal study was conducted in public and private laboratories of Fez-Meknes Hospitals, in order to collect information

about milk sensitivity as well as blood serum samples. Before any serum sample taking, a questionnaire was carefully completed with each patient, and a formal consent of each patient or of the children's parents was signed. The questionnaire contains data relating to age, sex and if there were any possible reaction to milk. Then, the collected sera were centrifuged at 3000 rpm/5 min and stored at -20 °C until use. The patients had not been sensitized beforehand with regards to milk proteins. They were patients who came for different medical tests, and they accepted to participate in the study benevolently. This study was approved by the ethic committee of the University Hospital Center of Fez.

Extraction of α-lactalbumin

The extraction of α-lactalbumin was realized according to Wal et al. (1995) (5) with some adjustments. In fact, a volume of 100 ml of raw 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 fraction was extracted in the supernatant, and dialyzed against bi-distilled water. The dialyzed extract was separated using gel filtration (G-100 Sephadex) column and the absorbance of fractions (50 fractions; 2 ml per tube) was determinated by an UV-Visible Spectrometer at 280 nm. The fraction presenting *α-LA* was then concentrated in a 10% polyethylene glycol solution (PEG). The quality of protein extracted was characterized by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE of α-lactalbumin

SDS-PAGE was performed under denaturation conditions in 20% polyacrylamide gel. A volume of 100 **µl** of the purified α-LA was mixed with loading buffer (10% SDS, 10% glycerol, 10% β-mercaptoethanol, and 2.5% bromphenol blue) and heated at 100 °C for 5 min. Then, the gel was fixed and stained using Coomassie Brilliant Blue R-250 (0.1%).

Dot-blot assay

Dot-blot assay was realized as described before (3). Briefly, 5 µl of purified α-LA was spotted on nitrocellulose membranes and incubated at 37 °C for 2 hours. Then, the dried spotted membranes were saturated by borate buffered saline (BBS) containing 2.5% Tween-20 for 1 h at 37 °C, in order to block the non-specific binding sites. Afterward, the membranes were incubated with human sera overnight at 4 °C, and later with anti-IgE peroxidase conjugate for 1 h at 37 °C. Finally, the reaction was revealed by the incubation of membranes in a solution containing 0.05% of diaminobenzidine (DAB) in BBS tampon. As indication, after each incubation step, the membranes were washed 3 times by BBS containing 0.1% Tween-20.

Heat-treatment and pepsin hydrolysis

The treatment of α-LA was performed on three sets of experiments; the first one was heat-treatment conducted in a thermostatic water bath (70, 80, and 90 °C) for 30, 60, and 120 min, the second one was pepsin hydrolysis (hog stomach, 3354 U/mg) at a concentration of 50 µg/ml in an acidic medium (pH = 2) during 30, 60 and 120 min at 37 °C, and the third one was the combination of the two treatments, heat-treatment followed by enzymatic digestion.

Production of polyclonal antibodies anti α-lactalbumin

Anti α-LA antibodies were prepared by immunizing rabbits against the native protein (α-LA) using Freund adjuvant. After five weeks, animals were sacrificed according to National Ethical Laws and blood samples were collected in dry tubes. After centrifugation for 15 minutes at 3000 rpm at 4 °C, serum was separated and frozen at -20 °C until use.

Specific IgE determination

In order to determine levels of specific IgE to milk and α-LA, indirect ELISA was used as described beforehand (17,18,4). Firstly, 100 µl of skimmed raw milk or α-LA (0.5 mg/ml) in PBS (Phosphate Buffered Saline, pH 7.4) was deposited on the wells of micro-titration plate (100 µl/well). Next, wells were saturated by BBS (borate buffered saline, pH 8.4) containing 2.5% Tween 20, and 100 µl of the human serum added. The revelation was made by adding the anti-human IgE conjugated to peroxidase, followed by addition of the ortho-phenylenediamine (OPD 0.05%) substrate. After incubation at 37 °C during 20 min, the reaction was stopped by adding 50 µl of HCl (3 mol/l) and the absorbance was measured at 490 nm by an ELISA reader.

Statistical analysis

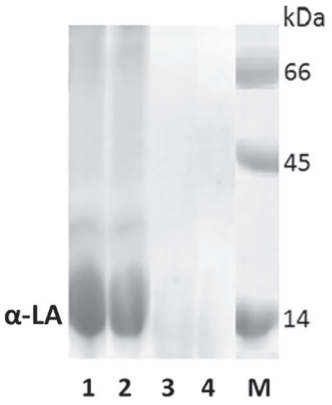
Descriptive statistics were presented as numbers with percentages or as average values. Statistical analysis was based on the student's t-test taking $p < 0.05$ as the limit of significant value. All statistical analyses were performed using Excel software.

Results

The questionnaire was fulfilled in by 832 subjects, represented by 54.5% of men and 45.4% of women. The age of the studied population ranged between 2 and 60 years old, among whom 18.8% were children (2-20 years) and 80.2% were adults (20-60 years).

Adverse reaction to milk was reported by 3.6% of the studied population, where children (2-10 years) and adults (20-40

Figure 1 *- Electrophoresis profile of α-lactalbumin. 1, native α-LA; 2, α-LA processed by heat-treatment; 3, α-LA hydrolyzed by pepsin; 4, α-LA treated by heat followed by pepsin hydrolysis. M, Molecular weight marker.*



years) were the populations reporting most sensitivity to milk, with 4% and 4.3%, respectively. The clinical signs mostly reported by our studied population were gastrointestinal reactions (73%), followed by cutaneous reactions (13%) and respiratory symptoms (6.6%).

The dosage of specific IgE to raw cow milk showed that 54.4% (303/557) presented positive values ranging from 2.7 to 595.2 IU/ml, with an average of 95.2 IU/ml. Among this population, 17.2% (n = 96) presented values more than 100 IU/ml, 6.6% (n = 37) more than 200 IU/ml and 4.6% (n = 25) more than 250 IU/ml. For adults, the average of IgE levels was 101.2 IU/ml, ranging from 1.91 IU/ml to 595.25 IU/ml, while the children population presented an average of IgE levels of 85.3 IU/ml, ranging from 2.75 IU/ml to 557.75 IU/ml. Regarding gender, the average of positive values of specific IgE levels was approximately the same; 97.96 IU/ml represented by men and 94.87 IU/ml represented by women.

Electrophesis of α-lactalbumin

The results of extracted α-LA native and treated by different treatments were presented in **figure 1**. The band of α-LA corresponded to a molecular weight of 14 kDa. The treatment of this protein by heat at 90 °C for 1 hour showed a very slight reduction in its band, but when it underwent pepsin hydrolysis for 1 hour with or without previous heating, the band totally disappeared.

Effect of heat-treatment and pepsin hydrolysis on the detection of α-LA by rabbit IgG by means of ELISA and Dot-blot assay

In order to determine the parameters of reduction of the immunoreactivity of α-LA to specific antibodies, we firstly studied its recognition by rabbit IgG anti-α-LA under heat-treatment, pepsin hydrolysis and under their combination. Under treatment by temperature (**figure 2**), the detection of α-LA was reduced after heating within 30 min at different temperatures, and was slightly modified for more heating time. Temperatures of 80 °C and 90 °C highly changed the liaison to IgG, more than 70 °C. Maximal reduction of IgG binding to α-LA were: 74% at 70 °C, 94% at 80 °C, and 97% at 90 °C. Concerning the hydrolysis by pepsin, we noticed that the detection of α-LA decreased progressively, until it reached a rate of 62% of decrease after 120 min of hydrolysis. While, when the two treatments were used, the detection of this protein by IgG was highly

Figure 2 *- Effect of heat-treatment on α-LA binding to rabbit IgG.*

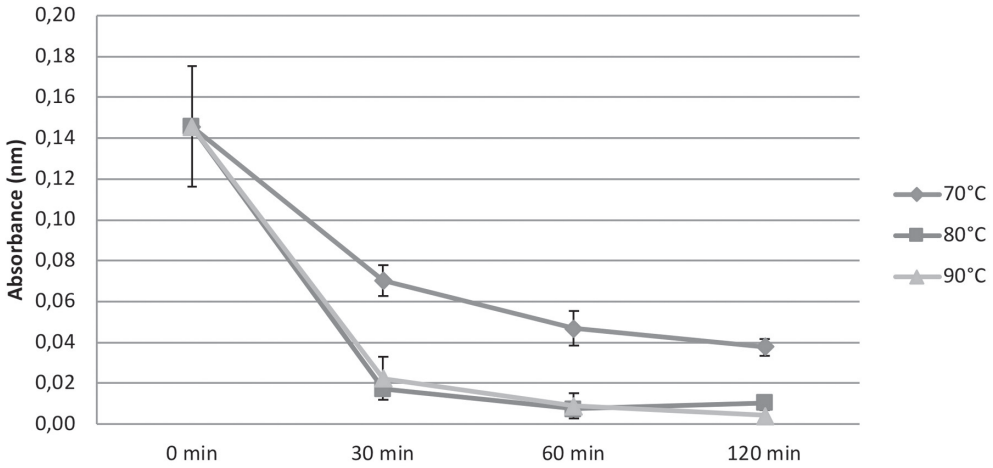


Figure 3 *- Effect of heat-treatment and pepsin hydrolysis on the recognition of α-LA by rabbit IgG.*

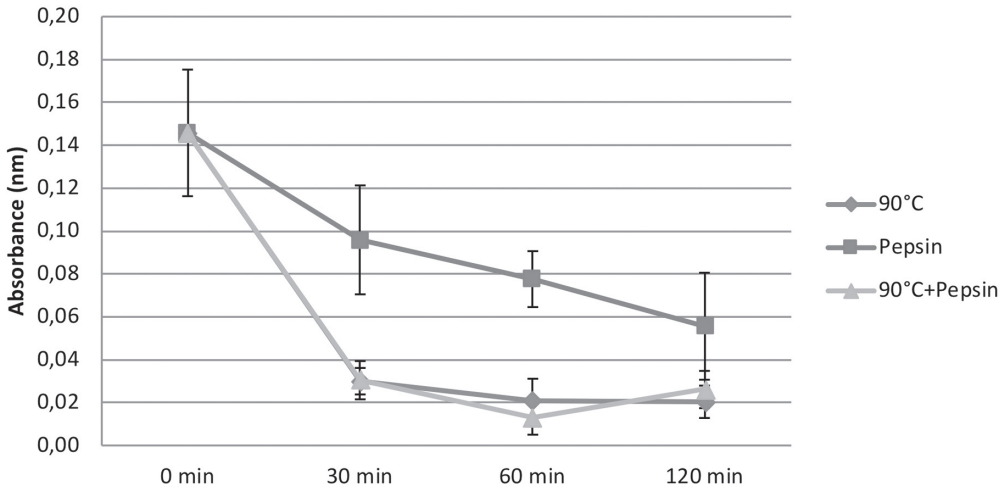
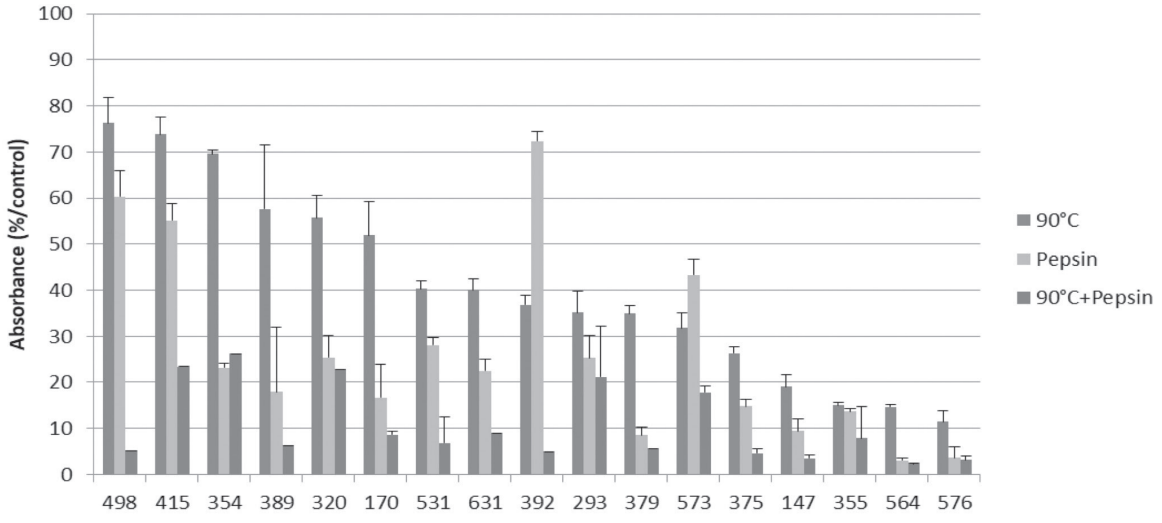


Figure 4 *- Dot-blot assay of α-LA with rabbit IgG anti native α-lactalbumin. 1, Dot-blot of native α-LA; 2, Dot-blot of α-LA processed by heat-treatment; 3, Dot-blot of α-LA treated by pepsin; 4, Dot-blot of α-LA treated by heat followed by pepsin hydrolysis.*

attenuated within 60 min of treatment, reaching a maximum of 91% of reduction (**figure 3**).

Similar results were observed using the Dot-blot assay. The presence of the blotting spot indicated that native α-LA reacted to IgG. However, when α-LA underwent different treatments for 60 min each, its recognition by IgG was modified. This modification was slight under heat-treatment for 60 min, while it was more important under pepsin hydrolysis as well as under the combination of treatments (**figure 4**).

Figure 5 *- Effect of heat-treatment and pepsin hydrolysis on the recognition of α-LA by human IgE.*



Effect of heat-treatment and pepsin hydrolysis on the detection of α-LA by human IgE by means of ELISA and Dot-blot assay

Sera of 17 patients presenting high levels of IgE to α-LA (> 90 IU/ml) were used to study the effect of treatments on the recognition of α-LA by human IgE basing on ELISA. The results showed that under heat-treatment at 90 °C during 60 min, all patients showed an important decrease in the recognition of heated α-LA. This reduction was more than 50% for 11 patients, with a maximum of 88% and an average of reduction of 59%.

Under pepsin hydrolysis, the results showed that all patients presented high reduction in the recognition of treated α-LA. This reduction was varying from 27% to 96%, and it was more than 40% for 82% of studied patients (n = 14 from 17). The average of this reduction was 74%.

In the same manner, high decrease reactivity to treated α-LA by a combination of heat-treatment and pepsin hydrolysis was observed for all studied patients, with an average of 89% and a maximum of 97%. Also, 82% of patients presented a significant decrease in the recognition of α-LA when the treatments were combined more than each one solely (**figure 4**).

Basing on Dot-blot assay, sera of 14 patients with high specific IgE levels to α-LA (> 65 IU/ml) were tested. The presence of spots indicated that all these patients reacted to native α-LA. Thus, their sera were used to study their reactivity to treated α-LA. The results showed that the intensity of the spots highly decreased when the protein was processed by heat-treatment for almost all patients, except two of them (14 and 17) still reacting to heated α-LA. As regards the pepsin hydrolysis and the combination of the two treatments, we remarked an absence of spots under the two treatments, indicating that the allergenicity of α-LA was strongly attenuated for all studied subjects (**figure 6**).

Discussion

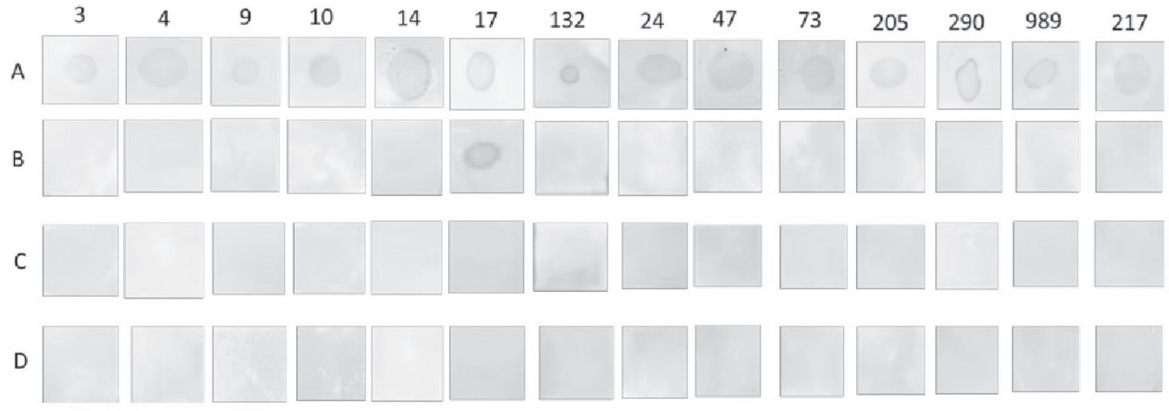
This research aimed to determine the effect of thermal treatment and enzymatic hydrolysis on the allergenicity of α-lactalbumin in a population from Fez-Meknes region of Morocco using ELISA and Dot-blot assay.

Our results showed that 3.6% of our population reported sensitivity to milk. We remarked that milk allergy was mostly reported in our studied population by children of less than 10 years and by adults of 20-40 years old. This result is in accordance with previous works concerning food allergy in Morocco, where our team research was involved (2,3,4,19).

Regarding specific IgE levels to cow milk, the results showed a strong sensitivity of the studied population to cow milk, especially in adult population aged 20-40 years. Therefore, we screened 31 sera sample presenting high levels of specific IgE to cow milk α-LA, in order to study the effect of thermal and enzymatic treatments on the allergenicity of α-lactalbumin.

Results showed that treatment by temperature reduced slightly α-LA band profile, while we observed by ELISA a high reduction in the IgE binding to heated α-LA for 70% of tested patients. This reduction presented an average of 59%, and varied from 23% to 88%. This result was confirmed by Dot-blot assay. This finding suggests that our studied population presented mostly conformational epitopes which has been reported to be denaturized under heat-treatment (20,21,22,4). Our results showed that the allergenicity of α-LA was decreased in all patients, and this decrease varied between 11% and 80%, indicating that there is a residual reactivity to heated α-LA persisted in some patients. This result of decreased allergenicity was in line with works of Bloom et al. (2014) (23) and Xu et al. (2015) (24), while the important residual allergenicity found is in ac-

Figure 6 *- Results of Dot-blot assay with sera of 14 patients. A, Dot-blot of native α-LA; B, Dot-blot of α-LA processed by heat-treatment; C, Dot-blot of α-LA treated by pepsin; D, Dot-blot of α-LA treated by heat followed by pepsin hydrolysis.*



cordance with a study of Lee et al. (2014) (25). This difference in results might be explained by the differences in studied subjects, as well as by heterogeneity in the method of study as these previous works based on pooled sera, while our study was done using individual sera and purified α-LA.

The results of pepsin hydrolysis exhibited the disappearance of α-LA band in SDS-PAGE profile, accompanied with an important decrease in rabbit IgG binding as well as in human IgE binding antibodies. This finding showed that α-LA lost its allergenic effect under pepsin hydrolysis, as it was reported in previous works (26,22,4). Regarding the antigenicity of hydrolyzed α-LA, our result was in line with the study of Kim et al. (2007) (27) who found that the antigenicity of α-LA decreased significantly when the concentrate of whey proteins was hydrolyzed by pepsin. Concerning the treatment by heat-treatment followed by pepsin hydrolysis, the binding to IgE antibodies was totally attenuated. This attenuation reached a maximum of 97% of the protein allergenicity. All studied subjects showed a significant decrease in IgE binding to treated α-LA under the combination of treatments which was more predominant than under each treatment solely (heat or hydrolysis). This indicated that the pre-heating may ameliorate the enzymatic action, as was reported previously (28).

These findings indicate that the majority of studied subjects recognize conformational epitopes, as there was an important decrease in the IgE binding after heat-treatment, while some patients showed slight decrease in IgE binding indicating that they recognize mostly linear epitopes. Furthermore, the pepsin hydrolysis alone or preceded by heat-treatment, caused an important decrease in the recognition of α-LA for all studied subjects. However, in previous works of our laboratory, the pepsin hydrolysis preceded by heat showed an apparition of new epitopes (18,2,3,22,4).

Conclusion

In conclusion, our study focused on the effect of heat-treatment and pepsin hydrolysis on the allergenicity of cow milk α-LA as one of allergens incriminated in milk allergy. The results showed that milk allergy could be related to α-LA sensitivity. We observed that there was a significant decrease in the α-LA allergenicity after heating and with hydrolyzed α-LA in all studied subjects. This indicated the implication of conformational epitopes in this allergenicity. Furthermore, the residual reactivity of IgE to heated α-LA, indicated that sequential epitopes were also implicated in the sensitivity of this population, but at less level.

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Conflict of interest

The authors declare that they have no conflict of interest.

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