

Molecular characterization of Amandin, an Allergen from Almond (*Prunus dulcis*) and the Effect of Heat and Enzymatic Treatments on Human IgE and Rabbit IgG Sensitivity to Almond Allergens

Bademde (*Prunus dulcis*) Bulunan Bir Allerjen Olan Amandin'in Moleküler Olarak Tanımlanması ve Enzim Uygulaması ve Isının Badem Hassasiyeti ile Oluşan İnsan IgE'si ve Tavşan IgG'sine Olan Etkisi

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Abstract

Background: Several clinical studies have shown that the allergy to nuts has increased significantly in recent years. Almond allergy is one of the most significant allergies in several countries. For this reason, we were interested in studying almond sensitivity, and especially one of its allergens: amandin.

Method: This work is based on a sample of sera from 252 patients. The samples were collected Ibn Elkhatib University Hospital Center and several private medical laboratories in Fez, Morocco. We also evaluated the sensitivity and immunoreactivity of human IgE and rabbit IgG to almonds variety of *Prunus dulcis* and investigated the effect of food processing (heat and/or enzymatic hydrolysis by pepsin) on this sensitivity. In addition molecular profile of amandin was studied.

Results: Reported allergy revealed that 6.5% of patients had an allergy to egg, whereas 2.5, 0.4, 7.8, 3.9, 2.5 and 2.2% of patients were allergic to peanut, wheat flour, fish, strawberry, milk and almond respectively. The evaluation of specific IgE showed that the study population is sensitive to the species *Prunus dulcis*. The study of the variation of allergenicity of almond under the effect of the temperature and the enzymatic hydrolysis showed an important decrease for the recognition of proteins by human IgE. However, enzymatic hydrolysis modifies the same way the binding of human IgE to almond inducing a reduction of the allergenicity. The molecular characterization of the allergens indicated a major reactivity to Pru du 6 (amandin). This reactivity is highly reduced by treatments, especially the enzymatic one.

Conclusion: Almond allergy could be reduced by heat treatment or enzymatic hydrolysis.

Key words: Almond protein, specific IgE, thermal treatment, acid treatment, amandin

Öz

Giriş: Çalışmalar, fındığa karşı olan allerjinin son yıllarda arttığını göstermiştir. Bazı ülkelerde ise, en önemli allerji bademe karşı olan allerjidir. Bu nedenle, biz de bademe karşı olan, özellikle de içindeki allerjenlerden biri olan amandine karşı olan allerjiyi çalışmak istedik.

Yöntem: Çalışma, 252 kişiden alınan kanların serumu ile yapıldı. Örnekler, Cezayir'in Fez şehrinde bulunan Ibn Elkhatib Üniversitesi Hastanesi ve bazı özel laboratuvarlardan alındı. Çalışmamızda ayrıca, çeşitli *Prunus dulcis* bademlerine karşı gelişen insan IgE'si ve tavşan IgG'sinin hassasiyetini, immün-etkileşimini ve bu etkileşime, yiyeceğe uygulanan ısı ve/veya pepsin ile hidroliz işleminin etkisini inceledik. Ayrıca, amandinin moleküler özelliklerini çalıştık.

Bulgular: Hastaların %6,5'inin yumurta allerjisine sahip olduğunu, bunların %2,5, %0,4, %7,8, %3,9, %2,5 ve %2,2'sinin sırasıyla yer fıstığı, buğday unu, balık, çilek, süt ve badem için alerjik olduğu saptandı. Özgün IgE'yi çalıştığımızda, toplumun *Prunus dulcis* çeşitlerine allerjisi olduğu saptandı. Bu allerjide, bademe karşı olan IgE'nin, ısı ve enzimatik işlemler ile azaldığı bulundu. Bununla birlikte, enzimatik hidroliz de aynı şekilde insan IgE'sinin bademe bağlanmasını modifiye ederek allerjenikliğin azalmasına neden olmaktadır. Allerjenlerin moleküler tanımlaması ile ana etkileşimin Pru du 6'ya (amandin) olduğu görüldü. Bu proteine olan etkileşim, ürüne uygulanan işlemlerden özellikle enzim eklenmesi ile önemli ölçüde azaldı.

Sonuç: Badem allerjisi, ısı işlem ve enzim uygulanması ile oldukça azaltılabilir.

Anahtar Kelimeler: Almond protein, özel IgE, termal ısı, asit tedavisi, amandin

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Introduction

Food allergy affects as many as 6% of young children and 3–4% of adults.^[1,2] During the past decade, concerns over avoidable mortality associated with allergic reactions to nuts has increased.^[3,4] The most potent allergens of plant origin include legumes, nuts, and seeds. The proportion of self-reported allergy to peanuts and tree nuts was about 0.4% in adults.^[5] A clear indication of the seriousness of tree nut allergies is the large percentage of fatal allergic reactions to foods for individuals over the age of six years.^[4]

There are four allergens of almond *Prunus dulcis*, Pru du 3 (Non-specific lipid transfer protein 1: nsLPT1; 9KDa), Pru du 4 (Profilin, 14 KDa), Pru du 5 (Acid ribosomal protein P2 60S; 10 KDa) and Pru du 6 (amandin, 11S globulin legumin-like protein; 360 KDa).^[6]

Frequency of nuts, in episodes of food-induced anaphylaxis varies. In France, for adults and children (n=60), an Almond allergy of 1.7% was reported^[7]; in Great Britain, for adults and children (n=90), allergy to almond was 3.3%^[8]; whereas the prevalences were 6.8, 2.3 for brazilian nut and hazelnut respectively in Spain^[9]; almond allergy was reported to be 5.6% in people between 12 and 75 years old in United States.^[10]

Almond is known to contain as many as 188 different proteins^[4,11] of which amandin is the major protein and constitutes up to 65% of the total soluble fraction.^[12] Amandin has been identified as a major allergen.^[13] The allergenicity of amandin, measured by the capacity to bind human IgE, has been demonstrated for multiple almond species and cultivars.^[14] Amandin, is officially recognized as an allergen (Pru du 6) by the International Union of Immunological Societies-Allergen Nomenclature Subcommittee. Amandin is a hexamer and each monomer consists of two polypeptides: a 40–42 kDa acidic subunit and a 20–22 kDa basic subunit.^[4,14,15]

Various food processing techniques, described in the literatures such as heating, enzymatic digestion and pH, can influence the allergenic potential of food proteins. It can either increase or decrease the allergenicity of the food.^[16–18] The epitopes can be described all along the allergenic molecule or basically situated in a specific area. By altering the protein by heating or by another treatment, its immunogenicity will change depending on whether the epitopes are sequential or conformational.

Conformational changes have been correlated with decreased immunogenicity.

To this effect, our aim was to evaluate the allergy to Almond Protein (AP) of a large section of patients at the region of Fez in Morocco. We were interested to evaluate IgE-sensitivity to AP and its modulation by heat treatment or enzymatic digestion compared to specific rabbit IgG to native AP. This study was also concerned with molecular characterization of the allergenic profile of the patients, especially to amandin.

Patients and Methods

1. Chemicals

The products used for this study were:

- Anti-IgE human sera conjugated to peroxidase, Sigma-Chemical (St Louis, Mo, USA);
- Bovine serum albumin (BSA), Sigma-Chemical;
- Orthophénylènediamine (OPD), Acrosorganics;

Other chemicals products were from Sigma or Merck.

2. Patients and Serum Collection

A cross sectional study was conducted at the University Hospital Center (CHU) of Fez, Ibn Elkhatib Hospital and at several Medical Test Laboratories in Fez in order to collect serum samples. The collection of human sera was performed between January and May 2016, with the approval of the ethical committee of the CHU. The samples were randomly collected. It is important to note that the patients did not undergo any prior sensitivity to the AP, and that they came for various medical tests. With the consent of the patients, a blood sample was taken and put in a 3 ml dry tube without anti-coagulant. After collecting blood samples, centrifugation at 1252 g for 5 minutes allowed us to recover and separate the sera, which was then kept at -20°C until use. The chosen patients had not been sensitized to the AP, or challenged orally.

3. Preparation of Defatted Almond Flours and Treatment of the Almond Protein (AP)

Almond was finely ground. The obtained powder or flour was defatted with chloroform and then dried before protein extraction which was achieved by suspending the sample in PBS (phosphate buffer solution pH 7.4) at 20% (w/v). The mixture was stirred for two hours, filtered and then centrifuged at 704 g, 4°C for 15 min. The collected

supernatant, considered as native AP, was frozen at -20°C until its utilization.^[4,17,19]

The native AP was then treated in four different ways as follows:

- Heating at different temperatures (75, 85, and 100°C) for 30, 60, 90 and 120 min,
- Treatment in an acidic (pH 2) or basic (pH 11) medium for 30, 60, 90 and 120 min at 37°C ,^[20]
- Digesting by pepsin (hog stomach, 3354 U/mg) at a concentration of 50 $\mu\text{g}/\text{ml}$ in an acidic environment (pH 2)^[18] during 30 to 120 min at 37°C ,
- Processing by a combination of the two treatments (heating and enzymatic digestion).^[20-22]

4. Amandin Purification

The protocol described by Albillos^[4] was followed and defatted almond flour was extracted at room temperature for 1 h using deionized distilled water containing 0.02% NaN_3 (flour/ H_2O ratio 1:50). The mixture was stirred using an orbital shaker with vortexing at 10 min intervals. After centrifugation (6000 g for 10 min), the residue was re-extracted once again in the same conditions. The supernatants were pooled and filtered through a filter. The clear filtrate was refrigerated (4°C) overnight for 12–14 h, and the milky precipitate was recovered after centrifugation (12000 g for 20 min, at 4°C). The precipitate containing mostly amandin was then re-suspended in 0.01 M PBS (pH 7.4) and dialyzed against distilled water (24 h, 4°C , three changes), then frozen and stored at -20°C in airtight plastic bottles until required.^[4]

5. Production of Polyclonal Antibodies Against the AP

To study the immunoreactivity of antibodies to almond, IgG antibodies were prepared against native AP. These antibodies were obtained after the immunization of rabbits against the native AP using Freund adjuvant. The AP were injected subcutaneously at several points on the animal back in combination with complete Freund's adjuvant for the first injection and with incomplete Freund's adjuvant in subsequent immunizations at one week intervals. After one month, blood samples were collected in dry tubes. After centrifugation for 15 minutes at 704 g at 4°C , sodium azide 0.02% was added to the sera and then frozen at -20°C until use.^[17,21,22]

6. IgE Determinations

Total IgE was evaluated by direct ELISA as described before.^[21,23,24] Briefly, diluted human sera were placed in 96 micro-titration plate wells and incubated overnight at 4°C . The non-specific sites were saturated with bovine serum albumin (BSA) 0.25% (200 $\mu\text{l}/\text{well}$). 100 μl of human anti-IgE peroxidase conjugate was then added and immune complex revealed after addition of 0.05% of orthophenylenediamine (OPD). Absorbance was measured at 490 nm by an ELISA reader (Labsystems Multiskan MS).

To immobilize allergens, the proteins of the AP were diluted in a phosphate buffered saline (PBS) at the rate of 0.5 mg/ml and put in a volume of 100 μl in each well. The plates were incubated at 4°C for one night. After washing, the plates can be conserved at -20°C until they are used. Human sera were added afterwards; the plate went through the same stages described for the dosage of total IgE. Quantification of IgE was made using IgE standards (10, 30, 70 and 90 IU/ml) as published before.^[17,20,23,24] Positive and negative controls were included in each plate to check the specificity and sensitivity of each measure. The determination of specific Serum IgE was achieved without prior sensitization or provocation tests of the patients.

The binding of rabbit IgG to the AP was determined by indirect ELISA in the same way as described for the determination of specific IgE. For each serum, determination of IgE was repeated at least in duplicate.

7. Size-exclusion Chromatography

We achieved this chromatography to separate the AP. The protein extract was diluted to a concentration of 1 mg/ml in PBS buffer, pH 7.4 and submitted to a volume of 2 ml in a column filled with Sephadex gel G 100, then a titration was carried out in a number of 50 tubes of 2 ml. The reading is made in UV-visible spectrometer at 280 nm.

8. Polyacrylamide Gel Electrophoresis

Protein profiling was carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The proteins of almonds were separated by 12% (w/v) polyacrylamide gel electrophoresis under denaturing conditions. The samples (5 mg/ml) of native or treated proteins were denatured by boiling samples for three minutes in the presence of SDS 10%, β -mercaptoethanol 0.8% (denaturing conditions). The migration was carried out in electrophoresis chamber, Hoefer scientific

Table 1. Description of the studied population.

Total	n	%	Average age (years)	Age interval (years)
Children	15	6,2	14	05 -16
Men	83	33,3	31	16- 58
Women	152	60,5	36	15- 65

instruments (San Francisco, California, USA), under a 25 mA current (consort EV243, Belgium) and the gel was stained with 0.1% Coomassie blue R250.^[20,25]

9. Ethics

This study was approved by the ethics committee of the University Hospital Centre of Fez. All experiments using animals have been conducted according to national and international laws.

10. Statistical Analysis

Statistics analysis was based on the student's t-test taking $p=0.05$ as the limit of the significant value.

Results

1. Description of Sample

252 adults including 152 women (60.3%), 83 men (33%) and 17 children (6.7%) were evaluated. The average age was 31 years for men, 36 for women and 14 for children. Ages ranged from 16 to 58 years in men and from 15 to 65 in women. The age of pediatric population ranged from 5 to 16 (Table 1). The determination of total human IgE was carried and resulted in a rate between 10 and 344 UI/ml.

2. Reported Allergy

The questionnaire was completed by 582 students from different institutions of Fez (Faculty of Sciences and Technology, Faculty of Science Dhar EL Mehraz, Faculty of Letter Dhar EL Mehraz and High Institute of Nursing and Technical Health), among whom 45% were men and 55% were women, aged between 17 and 26 years. The total percentage of reported food allergy was 30.9%, which could be distributed as 7.8% to fish, 6.5% to eggs, 3.9% to peanut, 3.9% to strawberry, 2.5% to milk, 2% to nuts and 2.2% to almonds. Cutaneous manifestations represented 47.7%, while digestive and respiratory allergy were 21% and 29% respectively. Anaphylactic shock was reported in 2.2% of patients. (Table 2).

Table 2. Description of reported allergy among students at the Faculty in Fez (n =582)

	Total n=582	%
Sex	Women	55
	Man	45
Age	Average age	25
Residence	Urban	92
	Rural	8
Type of allergy	Food allergy	30.9
	Allergy to pollen	23.8
	Drug allergy	1.9
	Allergy to insect toxins	8.9
	Other allergy	19
Manifestations	Digestive symptoms	21
	Cutaneous manifestation	47.75
	Respiratory symptoms	29
	Anaphylactic shock	2.2

3. Measurement of specific Serum IgE

A sample of 252 human sera was used to study the variation of the sensitivity to AP. In 24% of patients (n=62/252) anti-AP IgE was found to be higher than 2 IU/mL. Among all patients 10.4% had a level between 20 and 80 IU/mL, 7.55% had between 80 and 120 IU/mL and whereas 5% had anti-AG IgE at a concentration between 120 and 200 IU/mL (Figure 1). The average titre was 42 ± 2 IU/mL (n=62) ranging from 2.4 to 334.8 IU/mL. Of adults, men showed an average value of 17.6 ± 1.3 IU/mL (n=21/99) with a maximum of 222.6 IU/mL, while women had an average value of 34.2 ± 1.2 IU/mL (n=33/153) with a maximum value of 334 IU/mL. Women had a statistically higher concentration of IgE ($p < 0.05$). Among children, only 2 cases were positive for specific IgE: a 5-year-old child with a specific IgE level of 76.9 IU/mL and a 10-year-old child with a 101 IU/mL level (others had negative values).

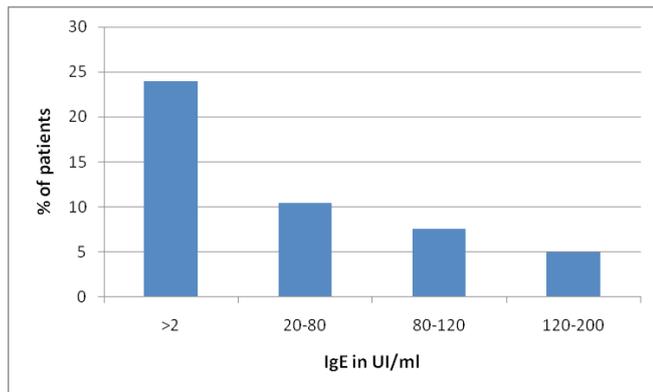


Figure 1. Distribution of specific IgE measured in patients in IU/ml.

4. Immunoreactive Rabbit IgG to Treated AP

To study the effect of heat and enzymatic digestion of AP, two sets of experimentation were conducted. The first examined the effect of temperature and enzymatic treatment on the detection of the proteins of the AP by a rabbit polyclonal antibody prepared at the laboratory through repeated immunizations by the native AP. The

objective was to identify the roles of temperature, pH, enzymatic hydrolysis with pepsin and the time sufficient to inhibit the reactivity of antibodies on the proteins of the AP. Figures 2 and 3 show the findings.

AP was heated at 70°C, 80°C, 90°C and 100°C at different time intervals varying from 30 min to 120 min. There was a decrease of polyclonal antibodies to AP at 30 min (Figure 2). The results showed a decrease of IgG binding to AP at all temperatures studied during the first 30 min of the treatment. At 100°C, the binding of polyclonal antibodies to these proteins was greatly diminished compared to the other two temperatures (70°C and 85°C).

During the first 30 minutes, the binding of polyclonal antibodies to AP was reduced by 20% at 85°C of temperature and 40% at 100°C. After 120 min, the binding of IgG with AP was decreased by 33% for the proteins heated at 75°C and 85°C and 52% when protein heated at 100°C.

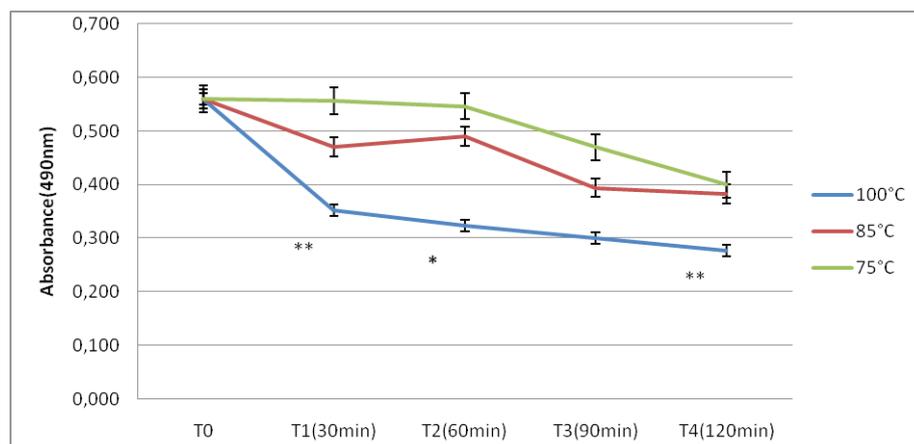


Figure 2. Effect of heat treatment on the recognition of AP by rabbit IgG. The effect of temperature (75°C, 85°C and 100°C) was studied in 4 experiments using rabbit IgG anti-native AP. The binding of IgG was evaluated by ELISA as the response of IgG binding to AP, heated at different temperatures over time. Compared to control value (at t=0 min), all values are statistically significant as $p < 0.05$. (n=4, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

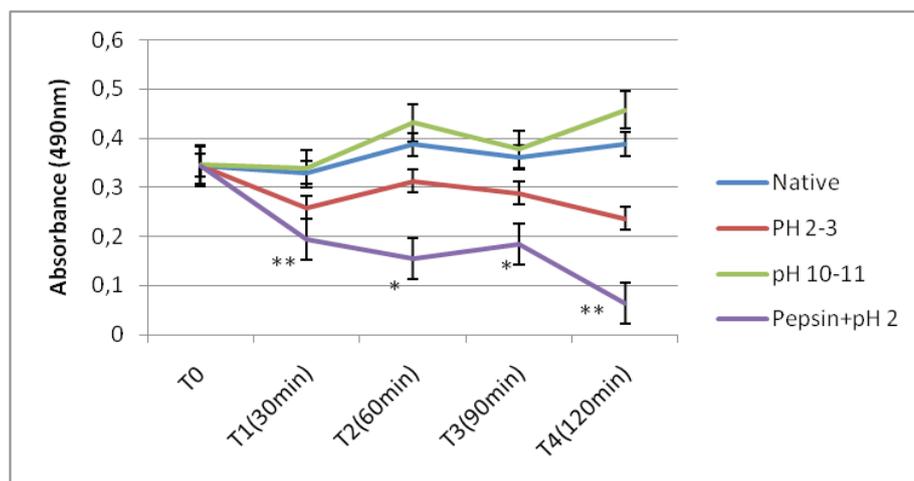


Figure 3. Effect of pH and enzymatic hydrolysis on the recognition of AP by rabbit IgG. The effect of these treatments was studied using rabbit IgG anti-native AP. The binding of IgG was evaluated by ELISA for determining the response of IgG binding to AP hydrolyzed in alkali, acid or by pepsin at 37°C over time. Compared to control value (at t=0 min), all values are statistically significant. (N=3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 3 demonstrates that the treatment of AP with pH 10–11 slightly increased the binding of rabbit IgG to AP during the first 60 min. IgG binding was decreased to the initial value after 90 min of treatment. The acidic pH decreased the binding of IgG to AP by 33% during 30 min. Pepsin hydrolysis in an acid environment greatly impaired the binding of IgG to AP which was reduced by 66% in the first 30 min of treatment. We observed a decrease of 91% of reactivity of the IgG after 120 min of treatment.

5. Human IgE Immunoreactivity to Treated AP

In the second set of experiments, we studied the variation of the allergenicity of the AP by using human sera whereby the rates of specific IgE rates have been proved to be important. This immunoreactivity was studied with regard to temperature, enzymatic treatment in different pHs.

For this reason, we have selected 20 patients with high IgE. These serums were used to determine the reactivity of

serum IgE to the proteins processed either at temperature 100°C during 30 min, or combination of heat treatment and pepsin digestion (Figure 4).

We found that the IgE binding to heated proteins was slightly diminished compared to that which has developed to native proteins (Figure 4).

Table 3 shows binding rates of anti-AP IgE to treated AP proteins. Recognition by IgE of the heated proteins decreased in 85% of patients. Among them, 61.9% showed a decrease less than 50%. Only one patient (5%) had increased binding.

IgE binding was decreased to heated and hydrolyzed AP. This decrease was observed in 95% of patients; however, no patients showed an increase in IgE binding, whereas only 1 patient (5%) showed no change from the native proteins.

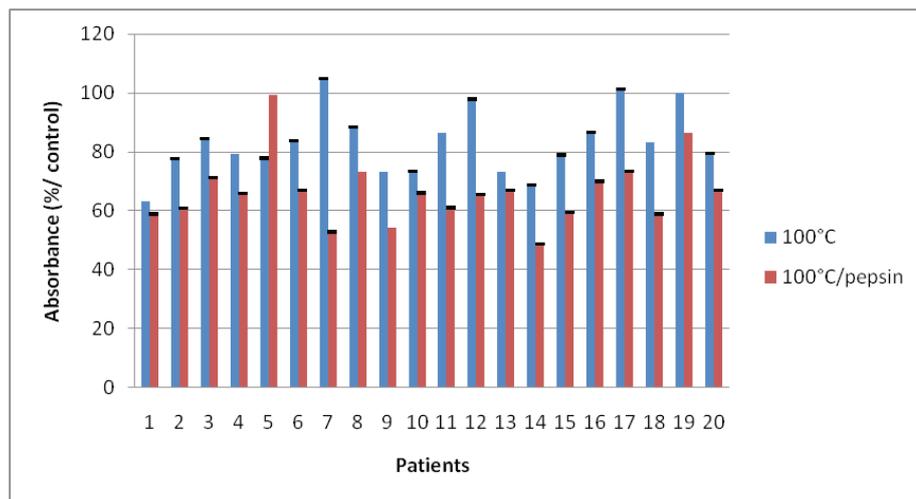


Figure 4. Effect of heating and enzymatic hydrolysis on the recognition of AP by human IgE. Absorbance represented percentage in relation to data obtained in control (Non treated AP). The control test was done by using native proteins. Values were obtained in the two different conditions, heating and combination of heating/pepsin hydrolysis treatments. In x-axis, numbers represent the laboratory identification number of patient’s sera.

Table 3. Ratio of variation of human IgE to AP treated by heating and combination of heating and enzymatic digestion

	Patients with increased binding	Patients with decreased binding	Patients with unchanged binding
AP heated/ Native	1	17	3
	5.0	85.0	10.0
AP heated and hydrolyzed/ Native	0	17	1
	0	95.0	5.0

6. Immunoreactivity of Boiled and Roasted AP

Raw foods underwent several transformations (heating, fermentation etc.) before they are distributed. Our goal was to compare the binding intensity of rabbit polyclonal antibodies to the proteins existing in various industrial products compared to the native proteins. The bindings of roasted and boiled almonds were studied.

Mean titre of polyclonal antibodies to roasted almonds decreased 72% compared to that was measured for native almond. (Figure 5).

7. Molecular Characterization and Electrophoretic Analysis

The size exclusion chromatography of almond (*Prunus dulcis*) is shown in Figure 6. In the protein extract precipitate, we were able to identify and separate the major allergen: the large molecule with a molecular weight of 360 kDa (amandin).

We also carried out an SDS-PAGE electrophoresis of differently processed AP. The protein composition of various extracts of AP (native, heated and/or treated with pepsin) was compared by polyacrylamide gel electrophoresis under denaturing conditions (Figure 7).

Three bands were seen corresponding to acidic subunit of amandin (40–42KDa), basic subunit of amandin (20–22KDa), and Pru du 4 or profilin (14 KDa) (Figure 7).

The migrated three bands correspond to 42, 22 and 14kDa.

The same profile was obtained at the protein processed by heating, and those processed by enzymatic hydrolysis. No band was found when the protein treated by heating followed by enzymatic hydrolysis.

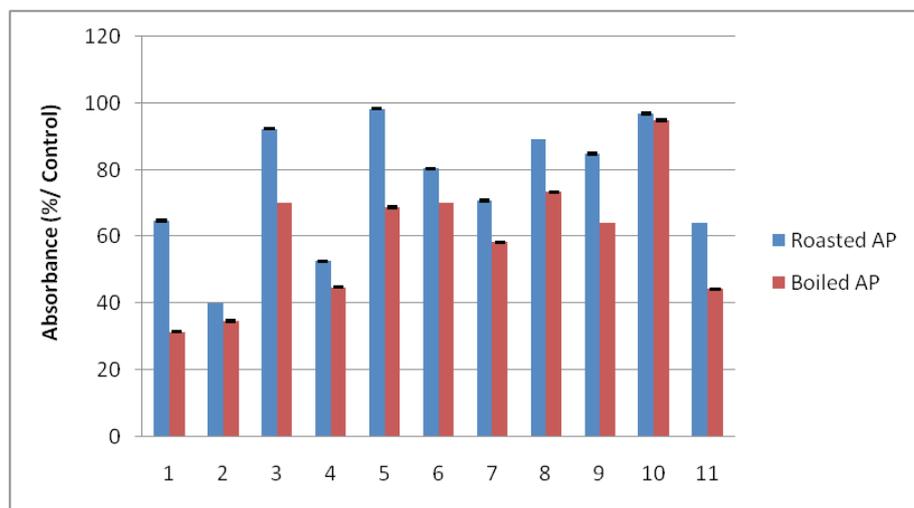


Figure 5. Graphical representation of Absorbance of IgG anti-AP boiled and roasted (n=11 tests). Absorbance represented percentage in relation to test values compared to data obtained in control (No treated AP). The control test was conducted by using native proteins.

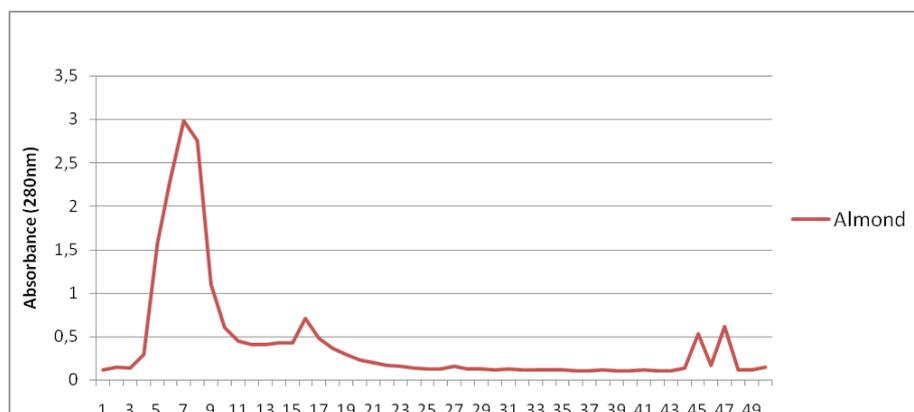


Figure 6. Size-exclusion Chromatography of Almond Protein Variety *Prunus dulcis*.

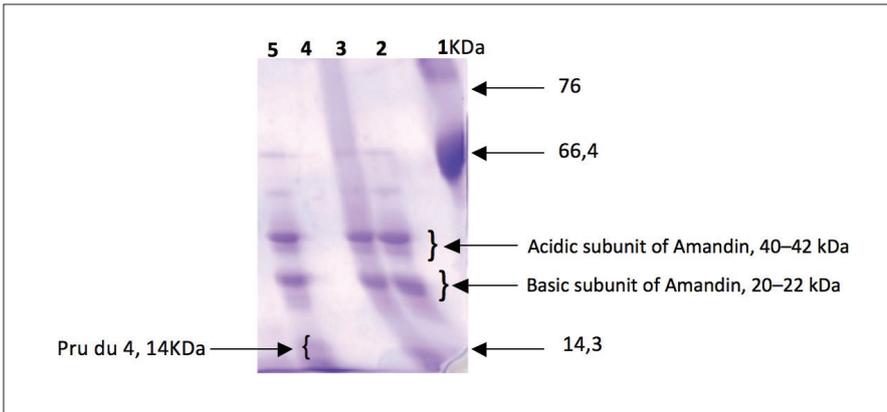


Figure 7. Electrophoresis profile of protein extracts of native almond (2); processed by heat (3); processed by heat and enzymatic hydrolysis (4); processed by enzymatic hydrolysis (5).

8. Sensitivity to Amandin

Figure 8 shows the serum level of anti-Amandin IgE of 20 patients in different conditions. Native amandin or those which were processed by heat or pepsin digestion/heat were used to evaluate their detection by the human serum IgE. There was a decrease of the immunoreactivity when the proteins were treated at 100°C. This decrease was even more pronounced with additional treatment of pepsin.

Discussion

Food allergies have significantly increased in the past few years, which reflects a major public health concern. The prevalence of food allergies is constantly rising.

The aim of our work was to study the characteristics of allergenicity to the AP among the population of the Fez region, as well as the effects of physicochemical treatment on these allergens.

Our methodology was based on the evaluation of the human IgE binding to the native proteins or the ones subject to thermal or enzymatic treatment. The biologic test used to evaluate these immunological reactions was ELISA, based on a set of human sera.

They reported that 2.2% of the students declared they have sensitivity to almond, which was higher than reported earlier in France (1.7%).^[7] Another study suggested that the frequency of nuts, in episodes of food-induced anaphylaxis showed different values in Great Britain with 3.3%^[8] and the USA with 5.6%.^[10]

A population of 252 patients was studied for the immunoreactivity against the AP. Among this population, 67% had a specific IgE level (against almond protein) higher than 50 IU/mL.

Among all patients 10.4% had a rate between 20 and 80 IU/mL, 7.55% between 80 and 120 IU/mL and 5%

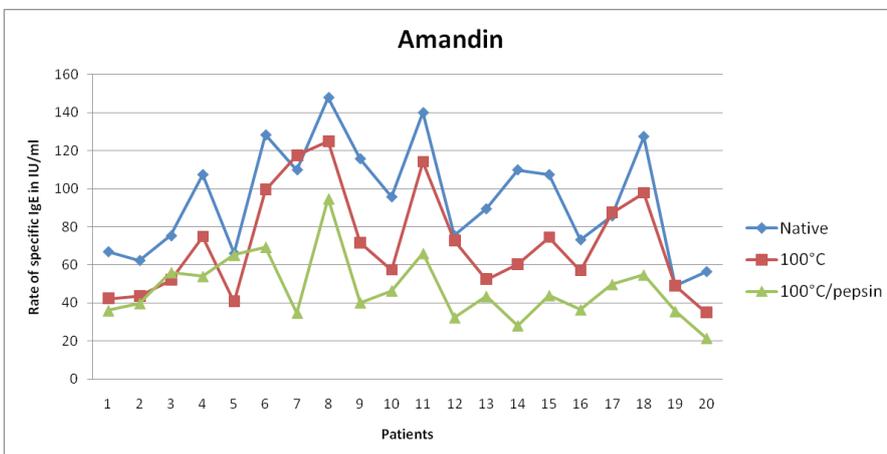


Figure 8. Serum level of specific IgE anti-amandin native and the effect of heating and enzymatic hydrolysis on the recognition of processed AP by human IgE.

between 120 and 200 IU/mL. The average concentration was 42 ± 2 IU/mL, and the minimum and maximum values ranged between 2.4 and 334.8 IU/mL. The IgE levels showed a strong sensitivity of the studied population to AP, which was explained by the daily consumption of almonds in Morocco.

The obtained results with rabbit IgG and IgE showed a reduction of the antibody binding for the heat-treated proteins compared to the native ones.

After a 120 min heat-treatment a 52% reduction of anti-AP IgG was noticed. Human IgE levels were decreased 85% when AP was heated. Decreased allergenicity with heat was observed for several allergens of plant of animal origin, which are known as thermolabile. These foods lose their allergenicity as shown for white beans^[18], peanut^[17] and wheat flour^[24,26], meats^[27], egg white^[20,28] and soy.^[29] These findings could be explained by a fragmentation of a part of proteins as suggested by the protein band attenuation observed by electrophoresis. The greater decrease observed for IgE in comparison with IgG suggested that some antigens recognized by IgE in the studied population had conformationally unstable antigenic sites, where, in contrast, a majority of antigens recognized by rabbit IgG had stable epitopes.

Another series of experiment were carried out pre-heated and/or enzymatically digested proteins. The enzymatic treatment was used to simulate the digestive enzymes on the food in the digestive tract, since all foods go through it before being absorbed.

Generally, the enzymatic hydrolysis known as a process which reduces the allergenicity of a protein and allows obtaining protein hydrolysates used in the formulas called "hypoallergenic".

The experiments for the almond proteins hydrolyzed with pepsin showed that anti-AP IgG has decreased by 91%, Similarly, anti-AP IgE was reduced at a rate of 95%. These numbers were higher than those obtained by heating only, without hydrolysis. This indicated that the pre-heating may ameliorate the enzymatic action.

Mondoulet et al.^[30-32] showed that the allergens of nuts, milk and peanut were sensitive to the hydrolysis by the digestive enzymes, such as the pepsin for the gastric digestion and the trypsin and chymotrypsin for the duodenal digestion.

Food processing can alter allergenicity of proteins, increasing or decreasing the potential to be recognized by immunoglobulin E (IgE).^[33,34] Albillos et al. (2009) found that amandin formed aggregates after heating in PBS at temperatures exceeding 77°C. These aggregates might bind to the antibody differently, compared with the native amandin and therefore cause a decrease in immunoreactivity. Wigotzki et al. (2000)^[35] investigated the stability of hazelnut protein extracts against various enzymes. They reported a decrease in the immunoreactivity of these proteins in different enzymatic environments.

We can speculate that the majority of epitopes recognized by both IgG and IgE are thermolabile, thus more conformational than sequential. However, a part of resistant sites to heat denaturation proves the existence of sequential epitopes.

Immunoreactivity of different industrial products was studied for the rabbit IgG. The immunoreactivity was evaluated for grilled and boiled food, and it was evaluated in two conditions: with or without enzymatic hydrolysis. In general, the IgG binding on these proteins decreases with hydrolysis time.^[36] The boiled foods showed the lowest immunological reactivity while the grilled ones had a high reactivity, indicating the development of new allergenic epitopes by Maillard reaction. As for the roasted foods, Müller found a strongly reduced IgE-binding to proteins from roasted hazelnuts.^[36]

The obtained results for the native amandin proteins (a major almond allergen) revealed the existence of 2 bands (Pru du 6, 11S globulin) which migrated around 40 and 42 kDa marking (Acid polypeptidic alpha chain), as well as the basic beta chain at 20 and 22 kDa. These results were comparable with other studies, suggesting a major reactivity for these two bands.^[25] The same electrophoretic profile was observed in various studies depicting these bands and their reactivity mainly with the human sera.^[4,37]

The same profile was obtained for the heat-treated proteins, and the ones having undergone the enzymatic hydrolysis. No band was revealed in the case of the protein extract treated with heat and followed by the enzymatic hydrolysis (combination of treatments). We can conclude that pre-heating releases the binding domains to the enzyme, which are originally unmasked.

Sensitivity to amandin is a common problem around the world. Our studied population had also a similarity

with the studied populations in the USA concerning the sensitivity to amandin.^[4,15] As for the diminution of immunoreactivity of antibodies after the heating of the amandin, it was previously detected. This indicates that the antigenic epitopes of amandin detected by the human IgE are predominantly conformational and are modified, at least partially, by heating.

CONFLICT OF INTEREST

We attest that all Authors listed on the title page have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission to this Journal.

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References

- Sicherer SH, Sampson HA. 9. Food allergy. *J Allergy Clin Immunol* 2006;117:S470-5. doi: 10.1016/j.jaci.2005.05.048
- Sampson HA, Mendelson L, Rosen JP. Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *N Engl J Med* 1992;327:380-4. doi: 10.1056/NEJM199208063270603
- Pumphrey RS, Stanworth SJ. The clinical spectrum of anaphylaxis in north-west England. *Clin Exp Allergy* 1996;26:1364-70.
- Albillos SM, Menhart N, Fu TJ. Structural stability of Amandin, a major allergen from almond (*Prunus dulcis*), and its acidic and basic polypeptides. *J Agric Food Chem* 2009;57:4698-705. doi: 10.1021/jf803977z
- Emmett SE, Angus FJ, Fry JS, Lee PN. Perceived prevalence of peanut allergy in Great Britain and its association with other atopic conditions and with peanut allergy in other household members. *Allergy* 1999;54:380-5(erratum 54:891).
- Allergen Nomenclature Sub-Committee, International Union of Immunological Societies(2001)Allergen Nomenclature: List of allergens. <http://www.allergen.org>
- Andre F, Andre C, Colin L, Cacaraci F, Cavagna S. Role of new allergens and of allergens consumption in the increased incidence of food sensitizations in France. *Toxicology* 1994;93:77-83.
- Pumphrey RS, Stanworth SJ. The clinical spectrum of anaphylaxis in north-west England *Clin Exp Allergy* 1996;26:1364-70.
- Novembre E, Cianferoni A, Bernardini R, Mugnaini L, Caffarelli C, Cavagna G, Giovane A, Vierucci A. Anaphylaxis in children: clinical and allergological features. *Pediatrics* 1998;101:E8.
- Kemp SF, Lockey RF, Wolf BL, Lieberman P. Anaphylaxis. A review of 266 cases. *Arch Intern Med* 1995;155:1749-54.
- Derbyshire E, Wright DJ, Boulter D. Legumin and vicilin, storage proteins of legume seeds. *Phytochemistry* 1976;15:3-24. doi: 10.1016/S0031-9422(00)89046-9
- Sathe SK, Wolf WJ, Roux KH, Teuber SS, Venkatachalam M, Sze-Tao KW. Biochemical characterization of amandin, the major storage protein in almond (*Prunus dulcis* L.). *J Agric Food Chem* 2002;50:4333-41.
- Roux KH, Teuber SS, Robotham JM, Sathe SK. Detection and stability of the major almond allergen in foods. *J Agric Food Chem* 2001;49:2131-6.
- Sathe SK, Teuber SS, Gradziel TM, Roux KH. Electrophoretic and immunological analyses of almond (*Prunus dulcis* L.) genotypes and hybrids. *J Agric Food Chem* 2001;49:2043-52.
- Albillos SM, Jin TC, Howard A, Zhang YZ, Kothary MH, Fu TJ. Purification, crystallization and preliminary X-ray characterization of prunin-1, a major component of the almond (*Prunus dulcis*) allergen amandin. *J Agric Food Chem* 2008;56:5352-8. doi: 10.1021/jf800529k
- Davis PJ, Smales CM, James DC. How can thermal processing modify the antigenicity of proteins? *Allergy* 2001;56 Suppl 67:56-60.
- Ouahidi I, Aarab L, Dutau G. Influence des traitements thermiques et acides sur l'allergénicité des protéines d'arachide au niveau de la population de la région Fès-Meknès au Maroc. *Rev Fr Allergol* 2010;50:15-21. doi: 10.1016/j.reval.2009.08.008
- Bousfiha A, Lotfi A. Effect of heat and enzymatic treatments on human IgE and rabbit IgG sensitivity to white bean allergens. *Iran J Allergy Asthma Immunol* 2013;12:304-11.
- Ouahidi I. Etude des sensibilités allergiques à différents aliments au niveau de la population Fès-Meknès: Epidémiologie, stabilité des allergènes aux traitements industriels et mise au point de trousse analytique. Thèse Doctorat Universitaire. Université Sidi Mohamed Ben Abdellah, 2011.
- Ouahidi I, El Youbi El Hamsas A, Aarab L. Modulation of egg white proteins allergenicity under physical and chemical treatments. *Food and Agri Immuno* 2011;22:57-68. doi: 10.1080/09540105.2010.526202
- Bousfiha A, Aarab L. Sensibilité de la population des régions de Fès et Casablanca au Maroc à la viande de poulet et effet de la température. *Immuno-analyse & Biologie Spécialisée* 2012;27:112-6. doi: 10.1016/j.immbio.2012.02.005
- Bousfiha A, Aarab L. Modulation of IgE immunoreactivity to broad bean proteins after food processing in a Moroccan population. *Allergol Immunopathol (Madr)* 2012;42:29-34. doi: 10.1016/j.aller.2012.06.004
- Ouahidi I, Elhamsas Ael Y, Bouyahyaoui Y, Mernissi FZ, Aarab L. Characterization and stability of specific IgE to white egg's, gliadin's and peanut's proteins among children. *Iran J Allergy Asthma Immunol* 2010;9:97-102. doi: 09.02/ijaa.97102
- Ouahidi I, Barakat A, El Hamsas AEY, Aarab L. Variation de l'immunoréactivité des IgE et des IgG vis-à-vis des gliadines sous l'effet du pH et de la température. *Immunoanalyse & Biologie Spécialisée* 2011;26:68-75. doi: 10.1016/j.immbio.2010.10.005
- L'Hocine L, Pitre M. Quantitative and qualitative optimization of allergen extraction from peanut and selected tree nuts. Part 2. Optimization of buffer and ionic strength using a full factorial experimental design. *Food Chem* 2016;194:820-7. doi: 10.1016/j.foodchem.2015.08.032
- Varjonen E, Björkstén F, Savolainen J. Stability of cereal allergens. *Clin Exp Allergy* 1996;26:436-43.
- Fiocchi A, Restani P, Riva E, Mirri GP, Santini I, Bernardo L, Galli CL. Heat treatment modifies the allergenicity of beef and bovine serum albumin. *Allergy* 1998;53:798-802.

28. Anet J, Back JF, Baker RS, Barnett D, Burley RW, Howden ME. Allergens in the white and yolk of hen's egg. A study of IgE binding by egg proteins. *Int Arch Allergy Immunol* 1985;77:364-71.
29. Besler M, Steinhart H, Paschke A. Stability of food allergens and allergenicity of processed foods. *J Chromatogr B Biomed Sci Appl* 2001;756:207-28.
30. Mondoulet L, Drumare MF, Leung SA, Paty E, Scheinmann P, Wal JM, et al. Influence des procédés thermiques sur l'allergénicité de l'arachide. *Rev Fr Allergol Immunol Clin* 2003;43:486-91.
31. Mondoulet L, Paty E, Drumare MF, et al. Influence of thermal processing on the allergenicity of peanut proteins. *J Agric Food Chem* 2005;53:4547-53. doi: 10.1021/jf050091p
32. Mondoulet L. Diversité de la réponse IgE dans l'allergie à l'arachide : caractérisation des allergènes et devenir de leur potentiel allergénique lors des traitements thermiques et des processus digestifs. Institut National des Sciences Appliquées de Toulouse 2005.
33. Cabanillas Martin B, Crespo JF, Burbano C, Rodríguez J. Systemic IgE-mediated reaction to a dietary slimming bar. *Ann Allergy Asthma Immunol* 2010;104:450.
34. Cuadrado C, Cabanillas B, Pedrosa MM, Varela A, Guillamón E, Muzquiz M, Burbano C. Influence of thermal processing on IgE reactivity to lentil and chickpea proteins. *Mol Nutr Food Res* 2009;53:1462-8. doi: 10.1002/mnfr.200800485
35. Wigotzki M, Schubert S, Steinhart H, Paschke A. Effects of in vitro digestion on the IgE-binding activity of proteins from hazelnuts (*Corylus avellana*). *Internet Symposium on Food Allergens* 2000;2:1-8.
36. Müller U, Lüttkopf D, Hoffmann A, Petersen A, Becker WM, Schocker F, Niggemann B, Altmann F, Kolarich D, Hausteil D, Vieths S. Allergens in raw and roasted hazelnuts (*Corylus avellana*) and their cross-reactivity to pollen. *Eur Food Res Technol* 2000;212:2-12. doi: 10.1007/s002170000245
37. Zhang Y, Zhang J, Sheng W, Wang S, Fu TJ. Effects of heat and high-pressure treatments on the solubility and immunoreactivity of almond proteins. *Food Chem* 2016;199:856-61. doi: 10.1016/j.foodchem.2015.12.063