STUDY OF SENSITIZATION TO APPLES AMONG MOROCCAN POPULATION IN FEZ REGION: ALLERGENIC PROFILE AND EFFECT OF HEAT AND ENZYMATIC TREATMENTS

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ABSTRACT

The apple, *Malus domesticus*, is a fruit that belongs to the Rosaceae family, and one of the most consumed fruit in the world. However, several clinical studies have shown that the problem of allergy to apples has increased significantly over the last years. On the one hand, our study aims at evaluating the sensitivity of the Moroccan population of the Fez region to three apple types: Golden Delicious, Starking Delicious and Granny Smith. On the other hand, we are interested in studying the influence of thermal and enzymatic treatments on the allergenicity of apple proteins. This work is based on a sample of sera of 73 patients of different sexes and age ranges. Sera were collected from the hospital Ibn Elkhatib of Fez University Hospital Center, and from private medical laboratories in Fez. Analysis of sera was performed by the ELISA technique for the measurement of apple specific IgE, as well as for the evaluation of the effects of heat and enzymatic treatments on the immuno-reactivity of the apple proteins. The reported study shows a low prevalence of allergy to apples among the Moroccan population. The results of measurement of IgE specific to apple proteins showed that the average of rate to specific of IgE was 55.8±3.3UI/ml to Golden Delicious. The study of the effect of thermal and enzymatic treatment shows that the Golden proteins lost most of their immune-reactivity with human IgE. The results obtained in this study open up an important perspective for allergic persons; the physicochemical treatment can be an alternative to heavy therapeutic treatment

Keywords: Food allergy, specific IgE, apple, thermal treatment, enzymatic hydrolysis.

INTRODUCTION

The apple (*Malus domesticus*) is one of the main fruit species that are cultivated in the world. Apples are not only eaten fresh; they are also used as ingredients in many food products such as jams, compotes, pastries and children formulas. However, several clinical studies have shown allergy to these fruits which increased substantially in recent years. The easy adaptability of the apple tree to different climatic conditions and resistance to low temperatures have enabled its cultivation in different climatic regions. It is a fruit that is consumed all over the world, and several apple types are marketed. Over 1000 apple types have been described, however, only a few are generally consumed[1]. In Morocco, the most widely cultivated variety is Golden Delicious; it represents more than half of the national production.

Although the Golden Delicious is demanding in cold, it has some adaptation ability allowing it to be grown in different conditions (Ministry of Agriculture and Fisheries Maritime: Strategy and Statistics Department).

According to the nomenclature of the World Health Organization Subcommittee, four major classes of allergens have been identified in the apple Mal d 1, 2, 3 and 4. The first three proteins belong to the family of PR or "Pathogenesis Related Protein" expressed in plants in response to stress, while Mal d 4 belongs to the family of profilin[2,3,4,5].

Food processing methods comprise a set of thermal and mechanical unit operations whose purpose is to structure, texture, and ensure adequate preservation of the food[6]. However, these methods may have different effects on the allergenicity of foods; they can cause the elimination of allergenic potential or the formation of new allergens.

This work aims at studying the sensitivity profile of a Moroccan population for the first time at the Fez region in Morocco to three types of apples: Golden Delicious, Red Delicious and Granny Smith, and assessing the thermal and enzymatic effects on the sensitivity of the apple proteins of the Golden Delicious.

This study is based on the determination of the recognition of human serum IgE with apple protein. In this context, a series of experiments were performed to investigate the immunoreactivity of human sera vis-à-vis the native potato proteins and those having undergone physico-chemical treatments such as thermal and enzymatic hydrolysis.

Patients and Methods

I. Apple Protein:

1. Protein Extraction:

The extract of food proteins is prepared from three types of fresh apple *Malus domesticus*: Golden Delicious, Red Delicious, and Granny Smith. The apples are carefully peeled, and the skin (protein rich parts) is finely ground using a mortar; the extraction is carried out by suspension of the apple skin in PBS buffer (phosphate buffer solution, pH 7.4) at 50 % (weight/volume). The mixture was stirred for two hours and then centrifuged at 7000 t/min for 20 min; then the supernatant is collected and stored at -20 ° C.

2. Protein treatment:

a. Thermal Processing:

Thermal processing of apple proteins was carried out by heating proteins at 90 ° C for one hour[7].

b. Enzymatic hydrolysis:

Enzymatic digestion of the proteins was performed by using pepsin (hog stomach, 3354U/mg, Sigma Aldrich USA) at a concentration of 50 µg/ml in an acidic environment (pH 2). The enzymatic hydrolysis was conducted at 37 ° C during one hour. The reaction is stopped by addition of NaOH[8, 9, 10].

c. Combination of treatments:

The native proteins were then processed by a combination of the two treatments (heating and enzymatic digestion). The protein extract is treated by heating at 90 ° C for one hour, then processed by pepsin in an acid medium and incubated at 37 ° C for one hour.

3. Survey study

A detailed questionnaire was distributed to a sample of 460 students from the FST (Faculty of Sciences and Technology) and the Faculty of Dhar Mehraz, which allowed us to collect information on age, sex, the presence or absence of an allergy, food responsible for allergy, as well as additional information.

4. Sera Collection

The collection of human sera was performed in 2015 from January to May with obtaining the approval of the ethic committee of Fez University Hospital Center (CHU). The individuals were selected at random; it is important to note that they have undergone no prior sensitivity vis-à-vis the apples. They came for various medical tests.

The sera collection was realized at the University Hospital Center (CHU) of Fez, the Hospital Ibn Elkhatib and private medical laboratories in Fez. Upon the consent of the patient, a blood sample was taken and put in 3ml dry tube without anti coagulant. After collecting blood samples, centrifugation of 4000 rpm for 5 minutes allowed us to recover and sera separated then keep at -20 ° C until use.

5. Serologic total IgE determinations

Human sera are incubated overnight at 4°C in a 96-well micro-titration plate. After washing for three times with BBS containing 0.1% of Tween-20 and 0.1% of bovine serum albumin (BSA), the plates are saturated with BSA (0.25%) for an hour at 37°C (200 ml/well). After removal of BSA and washing, the antibody human anti-IgE peroxydase conjugate was added (100 ml/well) in 2 hours at 37°C. The revelation of the immune complex is done after washing by adding chromogene, orthophenylenediamine (OPD) to the concentration of 0.05%. The reaction is stopped by adding HCl 3M. Absorbance was subsequently determined at 490 nm.

6. Specific IgE determinations

The protein extract was diluted to a concentration of 0.5 mg / ml in PBS buffer, pH 7.4 and put at a volume of 100 µl per well. After incubation for 40 min at 37 ° C and washing, three washes are performed with BBS Tween 20 (0.1%). Then the wells were saturated with bovine serum albumin (BSA) at 0.25 % in an amount of (200 µl per well). Then human sera were added and plates incubated for 40 min at 37 ° C. Human Ig E binding was revealed by adding anti-IgE conjugated to peroxidase followed by substrate OPD at 0.01% diluted with sodium citrate buffer (0.05 M, pH 5.1). The developed color reaction was stopped by 3 M HCl and the absorbance was measured at 490nm by an ELISA reader (Labsystems Multiskan MS)[11,12,13]. Quantification of IgE was made using IgE standards (10, 30, 70 and 90 IU/ml) as published before[7,9,12]. Positive and negative controls were included in each plate to check the specificity and sensitivity of each measure. The determination of specific serical IgE is achieved without prior sensitization or provocation tests of the patients.

7. Chemicals and reference substances

The products are:

• anti-IgE human sera conjugated to peroxidase, Sigma-Chemical ;
• bovine serum albumin (BSA), Sigma-Chemical ;
• orthophénylénediamine (OPD), Acros organics ;

Other chemicals are from Sigma Chemical or Merck.

8. Ethics
This study was approved by the ethics committee of University Hospital Centre of Fez.

9. Statistical analysis
Statistical analysis was based on the test of student. The test Khi2 analyze was used for statistical correlation determination.

RESULTS

Humain Serum IgE Dosage

1. Sample Description

The total number of patients enrolled was 73 patients, including 55 women (75.3%), 18 men (24.6%), among them there are 21 children (28.7%). Patients included in this study have high total IgE levels from 62 to 744 IU/ml and showed an average of 130.4 IU/ml and 160.7 IU/ml for children and adults, respectively. From them, 11 patients are aged between 0 and 10 years, 10 patients are aged between 10 and 20 years, 41 of them between 20 and 40 years, and 11 patients are over 40 years old.

2. Reported allergy
The questionnaire was completed by 460 students, 32% are men and 68% are women, aged between 17 and 30 years old; the percentage of reported total allergy is 22%. 5% are allergic to peanuts, 2%, to almonds, 6% to milk, 11% to eggs, 9% to fish, 8% to strawberry, and 0,65% to apples.

3. Sensitivity to apples proteins
From 73 sera collected to investigate the sensitivity to proteins of three apple types highly consumed in Morocco: Golden Delicious (GD), Red Delicious, and Granny Smith. The results of measurement of IgE specific to apple proteins showed that the average of rate to specific of IgE was 55.8±3.3UI/ml to GD, 34.9 IU/ml ± 4.09 IU/ml to the Red Delicious and 14.39 IU/ml±5.01IU/ml to the Granny Smith. We noted that the studied population was more sensitive to the Golden Delicious than other types of apples, for this reason, we were confined to the study of the sensitivity of this type.

The results of specific IgE have shown that 20.54 % of patients had specific IgE that is superior to 100UI/ml and 8.21 % of them have a rate between 50 and 100 UI/ml and 5.47% have values situated between 15 and 50 UI/ml. The average of these positive values of specific IgE is 55.8IU/ml ± 3.3IU/ml (n=73) varying between 0.7 IU/ml to 409 IU/ml (Table 1). For adults, specific IgE had an average of 62.5 IU/ml (n=52), ranging from 1.5 to 370 IU/ml. The specific IgE in children had an average of 64.8 IU/ml (n=21), ranging from 0,7 to 409 IU/ml.

4. Effect of heating and enzymatic treatment on Immunoreactivity of Human IgE
A sample of human sera of 24 patients was used to investigate the influence of thermal and enzymatic treatments on the sensitivity of Golden Delicious proteins. A serie of experiments was conducted to assess the recognition by human IgE of processed apple proteins. For that, golden proteins have been either by heating treatment (heating at 90°C for one hour) or by enzymatic hydrolysis with pepsin, and heat treatment followed by enzymatic hydrolysis with pepsin.

The results obtained on the graph figure 1 are converted in table 3, which represents in percentage the variation of the binding of human IgE to Golden proteins. Figure 1 shows the variation of the binding of human IgE to percentage processed apple proteins according to the ratio of the treated proteins on native proteins.

Based on obtained results, we observed a decrease in binding of IgE to apple protein treated by heating. Recognition of IgE to the heated proteins decreased in 87.5% of patients; 61.9% of them showed a percentage of binding below 50%. However, this recognition has increased in 3 patients (12.5%).

Concerning the enzymatic treatment, it is found that the recognition by IgE of hydrolysed apple protein decreased in the majority of patients (91.6%); 68.1% of which show a percentage of binding lower to 50 % However, this binding was stable in 2 patients (8.3 %). The increase of the hydrolyzed protein IgE binding was not observed in the patients studied.

With regard to the combination of thermal and enzymatic treatments, there is a decrease in binding of IgE to apple proteins heated and hydrolyzed. This decrease was observed in 83.3 % of patients. However, 2 patients (8.3 %) showed an increase in IgE recognition protein, and two other patients showed no change relative to the connecting native proteins (Table 2).

DISCUSSION

The prevalence of food allergy has increased significantly in recent years. Currently, food allergy is ranked fourth among the diseases in the world according to the World Health Organization. Among the possible causes of this increase we can mention changes in eating habits and the development of food processing techniques.

The objective of this study is to investigate the sensitivity of a Moroccan population of the region of Fez to apples, as well as evaluation the influence of the physicochemical treatment on the sensitivity of the apples.

The methodology is based on a survey conducted in the city of Fez followed by the creation of a bank of human sera. Sensitivity was tested by measurement of specific IgE to human serum in relation to apple proteins by the ELISA technique. Finally, we studied the immunoreactivity of human IgE to native apple proteins or subjected to heating or enzymatic treatments.

The survey revealed that 0,65% among the 460 surveyed people showed sensitivity to apples. The study of the sensitivity profile (IgE assay) of the Moroccan population to three types of apples: Golden Delicious, Red Delicious, and Granny Smith, showed that the studied people more sensitive to the Golden Delicious variety that Red Delicious followed by Granny Smith. The study of Bolhaar et al. (2005)[15] on the allergenicity of different varieties of apples on a sample of Dutch and Spanish patients showed that Dutch patients showed positive prick tests to the skin of Golden Delicious. Allergy to Rosaceae is rarely reported in the French pediatric series[16]. In 2002, data from the CICAA (circle of clinical and laboratory investigations food allergy) reported observations of six pediatric Rosaceae allergy with a frequency of 0.68% (17th position)[17]. The study of Rance et al. in 1999[18] collects three cases of allergy to apple, three cases of allergy almonds, and one case of fish allergy on a series of 544 children. In Spain, allergy to fruit arrives in 4th position (20.8%) from food allergies of children, which 62% are allergic to Rosaceae[19,20,16]. The Network's investigations allergovigilance of 2001

and 2002 identified a case of analog severe food prophylaxis with apple and two cases in Fishing [21, 22].

The results of the immunoreactivity of human IgE vis-a-vis apple proteins having undergone a heat treatment showed a significant decrease in the recognition of IgE vis-a-vis of these proteins. After heating the proteins at 90 ° C for one hour, their binding to human IgE were decreased in 87.5% of patients. These results correlate with those of a study which showed that heating the apple protein extract significantly reduced its immunoreactivity vis-a-vis IgE[23].

Recognition of IgE vis-a-vis the heated proteins increased in 3 patients; this may be explained by the fact that the heat treatment induced the appearance of new allergenic sites probably sequential to or resulting from reactions with other compounds such as Maillard reaction.
Another series of experiments was conducted to evaluate the effect of enzymatic digestion by pepsin on the allergenicity of Golden Delicious proteins. The immunoreactivity of IgE vis-a-vis hydrolyzed Golden protein revealed that the majority of patients (91.6%) showed a decrease in the recognition of IgE vis-a-vis these proteins. From these results it can be concluded that the enzymatic hydrolysis strongly reduced the allergenicity potential of these apple proteins.

During the combination of the heat and enzyme treatment, we noticed a decrease in the recognition of IgE vis-a-vis the heated and then hydrolysed proteins in 83.3% of patients. In addition, we noticed an increase in the IgE binding to treated proteins in 2 patients that are different from those that showed an increase by heating.

When the enzymatic hydrolysis is preceded by heating, there is an increase of IgE binding to the proteins in 2 patients. From these results it can be deduced that the Golden proteins were denatured by heating, resulting in new peptide epitopes recognized by the IgE, which are responsible for increasing the immunoreactivity of these proteins.

Food allergies represent a major public health problem because of their significant impact on morbidity, mortality and costs. Several epidemiological studies have shown an increased prevalence of food allergy over the last decades; this is due to changes in eating habits and changes in food processing technology and the emergence of new constituents used as food additives.

This work is a combination of epidemiological and biological studies. The results of the effects of physicochemical treatments showed that Golden Delicious proteins lose most of their allergenicity after treatment by heating or enzymatic hydrolysis. The study of the effects of food transformation processes (heat treatment, enzymatic digestion ...) on the allergenic activity of food, as well as the study of the effect of interactions between protein allergens and the ingredients used in manufactured products on assimilation and sensitivity to the allergen allow a better knowledge of allergens related to food technologies and a better understanding of allergic phenomena and the conditions of the food allergy risk.

The results obtained in this study open up an important perspective for allergic persons; the physicochemical treatment of certain foods can be an alternative to heavy therapeutic treatment.

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.

ACKNOWLEDGEMENTS

This work was supported by grants of the Laboratory of Bioactive Molecules (LMBSF), Faculty of Sciences & Techniques from Fez. We would like to thank the hospital Ibn Elkhatib of Fez, the University Hospital Center, and the private medical laboratories in Fez for their help in collecting sera.

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Table 1: Percentage of patients sensitive to Golden Delicious

Total Number of studied patients **Average of specific IgE**	**All 73 (100%)** **55.8UI/ml ± 3.36UI/ml**
Number of patients whose Specific IgE > 100UI/ml	15 (20.54%)
Number of patients whose Specific IgE is between 50-100 UI/ml	6 (8.21%)
Number of patients whose Specific IgE is between 15-50 UI/ml	4 (5.47%)

Table 2: Ratio of variation of human IgE to Golden Delicious proteins treated by heating, enzymatic digestion, and combination of the two treatments

	Heating at 90°C	**Enzymatic Digestion**	**Combination of the two treatments**
Number of patients with a % <90%	**21 (87.5%)**	**22 (91.6%)**	**20 (83.3%)**
Number of patients with a % 90-110%	**0 (0%)**	**2 (8,3%)**	**2 (8.3%)**
Number of patients with a %>110%	**3 (12.5%)**	**0 (0%)**	**2 (8.3%)**

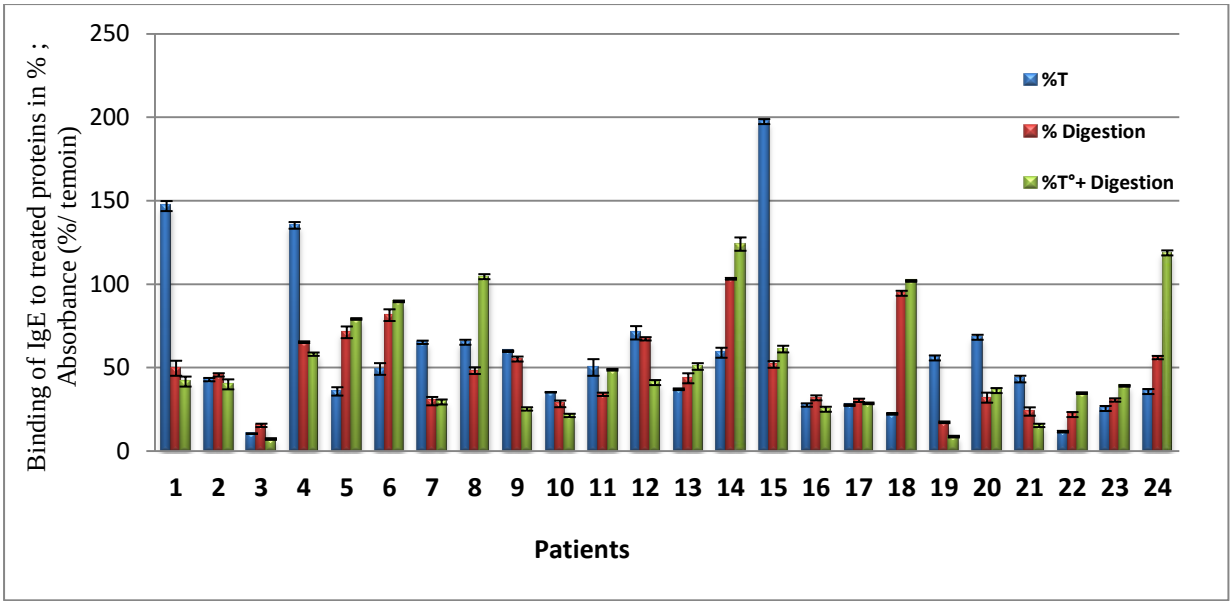

Figure 1 : Graphic Representation of the effect of heating, enzymatic digestion, and the combination of the two treatments on the recognition of human IgE in relation to Golden proteins.