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Effect of Heat and Enzymatic Treatments on Human IgE and Rabbit IgG Sensitivity to White Bean Allergens

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ABSTRACT

The aim of this study was to evaluate the sensitivity of the population of Fez and Casablanca in Morocco to dry white beans (*Phaseolus Vulgaris*) and to investigate the effect of food processing (heat and/or enzymatic hydrolysis by pepsin) on this sensitivity. Work was based on a bank consisting of 146 sera from patients with atopic hypersensitivity in order to evaluate specific immunoglobulin E (IgE) levels to native and processed white bean proteins by ELISA. Under the same conditions, we assessed the immunoreactivity of rabbit IgG obtained by immunization with native white bean proteins.

Evaluation of specific IgE to the white bean proteins showed that 51% of children and 39% of adults had positive values. The heat treatment and pepsin hydrolysis of dry bean proteins showed a reduction of 68% of IgE binding recognition in more than 65% of all patients. After heating, all patients indicated a reduction greater than 36%. With rabbit IgG, we observed a decrease by 25% of binding under heat treatment while enzymatic digestion reduced IgG recognition by 46.6%.

These findings suggest that epitopes recognized by IgE in the studied population were conformational sites whereas those recognized by rabbit IgG were probably sequential. In conclusion, these results demonstrate that the Moroccan population was very sensitive to white beans and this sensitivity could be reduced after heat treatment or enzymatic hydrolysis.

Keywords: Beans; Food allergy; Food processing; Immunoglobulin E; Pepsin.

INTRODUCTION

Food allergy is a prominent public health concern that affects both adults and children.

The prevalence of food allergy seems to be on the

rise. IgE mediated food allergies are reported between 3-4% in adults and 6% in children.¹ In Morocco, 9.5% of adults reported allergy to foods.² In atopic children, a sensitization to food was observed in 45%.³

Legumes are important food in the world including in Morocco. They are one of the main sources of protein and dietary supplement for the Moroccan population. The Papilionaceae family includes the most important allergenic species: *Lens culinaris* (lentil),

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Effect of Enzymatic Treatments on Sensitivity to White Bean Allergens

Cicer arietinum (chick-pea), *Pisum sativum* (pea), *Arachis hipogea* (peanut), *Phaseolus vulgaris* (bean) and *Glycine max* (soy). From this family, peanuts are identified as an important food causing allergies in Morocco.^{3,4} In Spain, lentils and chickpeas are the most frequent causes of children's allergic reactions to legumes^{5,6} while peanut and soy are the legumes most frequently involved in human food allergies in other countries such as the United States, the United Kingdom and Japan.^{7,8} Allergy to green beans was well established⁹⁻¹³ compared to the rare white dry beans allergy, that has involved severe allergic symptoms.¹⁴⁻¹⁶

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.^{2,17-20}

The aim of this study was to evaluate IgE-sensitivity of the population both of the Moroccan cities Fez and Casablanca to white bean proteins and its modulation by heat treatment or enzymatic digestion compared to specific rabbit IgG to dry beans.

PATIENTS AND METHODS

Chemicals

All chemicals products used in this study were from Sigma (St Louis, Mo, USA).

Patients

The work was conducted on a sera-bank, obtained from 146 volunteers, who were suffering from various symptoms, most of which were cutaneous (urticaria, angioedema) and respiratory disorders (rhinitis, asthma). These atopic patients were consulted for hypersensitivity, addressed by dermatology and pneumology services to laboratories in order to measure their total IgE.

After formal consent from each patient, a serum sample was collected from the University Hospital Center of Fez as well as from biomedical laboratories in Fez and Casablanca. The collected sera were stored at -20 °C until they were used. The patients recruited had not been sensitized to the white beans, nor challenged orally.

Extraction and Treatment of the white Bean Protein (WBP)

The seeds of beans were very finely ground. The

powder or flour obtained 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 3000 rpm for 15 min at 4°C. The collected supernatant, considered as native WBP, was frozen at -20°C until it was used.

The native WBP was then treated in four different ways. It was either (1) heated at different temperatures (70, 80, 90 and 100°C) for 60, 120 and 180 min, (2) treated in an acidic (pH 2) or basic (pH 11) medium for 60, 120, 180, and 210 min at 37°C, (3) digested by pepsin (hog stomach, 3354U/mg) at a concentration of 50 µg/ml in an acidic environment (pH 2) during 30 to 210 min at 37°C or (4) processed by a combination of the two treatments (heating and enzymatic digestion).

Production of Polyclonal Antibodies against the WBP

To study the immunoreactivity of antibodies to WBP, IgG antibodies were prepared against native WBP. These antibodies were obtained after the immunization of rabbits against the native WBP using Freund adjuvant.

The WBP 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, animals were sacrificed and blood samples were collected in dry tubes. After centrifugation for 15 minutes at 3000 rpm at 4°C, sodium azide 0.02% was added to the sera and then frozen at -20°C until use.

IgE Determinations

Total IgE was evaluated by direct ELISA as described before.^{2,21} 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% (200µl/ well). Then 100µl of human anti-IgE peroxidase conjugate was 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). Quantification of IgE was made using IgE standards (10, 30, 70 and 90 IU/ml) as published before.^{2,4,21} Positive and negative controls were included in each plate to check the specificity and sensitivity of each measure.

For specific IgE, indirect ELISA was used. The WBP diluted at 0.5 mg/ml in PBS (phosphate buffered saline) was deposited on the wells of micro-titration plate (100 µl/well). After overnight incubation at 4°C, wells were washed, saturated with bovine serum albumin 0.25% and the plate was treated. Human sera, diluted with BBS (borate buffered saline), were added afterwards; the plate went through the same process for the total IgE determination.

The binding of rabbit IgG (obtained as described before) to the WBP 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.

Polyacrylamide Gel Electrophoresis

The proteins of beans were separated by 12% (w/v) polyacrylamide gel electrophoresis under denaturing or non-denaturing conditions. The samples (5mg/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 done in electrophoresis chamber, Hoefer scientific instruments (San Francisco, California, USA), under a 25mA current (consort EV243, Belgium) and the gel was stained with 0.1% Coomassie blue R250.

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.

Statistical Analysis

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

RESULTS

Sample Description

The total number of patients recruited for this study was 146 patients; 98 children (67%) and 48 Adults (33%).

The pediatric population ranged from one to 15 years, with an average age of 7.5 years. Adults were composed of 73% of women (17 to 57 years with an average age of 35 years) and 27% of men (19 to 55 years with an average age of 37 years).

Distribution of IgE Levels

All patients recruited have been tested for total IgE. The total IgE varied from 0 to 70.55 IU/ml and showed an average of 13.3 IU/ml and 16.7 IU/ml for children and adults, respectively. Among 146 patients, 114 had been tested for specific IgE representing 78% of all children and 79% of all adults recruited.

The specific IgE in children had an average of 22 IU/ml, ranging from 2 to 68 IU/ml. Among 76 children, 39 (51%) had positive values (> 2 IU/ml), and 20% had higher levels of up to 20 IU/ml. For adults, the average of specific IgE was 19.3 IU/ml ranging from 2 to 37.1 IU/ml, among which, 15 (39%) had positive levels prevalent among 21% of men (8) and 18% (7) of women. Forty% of these adults, had levels of up to 20 IU/ml where 13% were men and 27% were women.

Immunoreactivity of Treated WBP with Rabbit IgG

The effect of temperature, pH and enzymatic digestion was studied on the immunoreactivity of WBP with rabbit IgG prepared against native WBP.

WBP were heated at 70°C, 80°C, 90°C and 100°C at different time intervals varying from 30 min to 180 min. Treated samples were then used for IgG binding evaluation using ELISA. The results (Figure 1) showed a decrease of 25% of IgG binding to WBP at all temperatures studied during the first hour of treatment after which this reduction did not increase further and remained relatively stable during the subsequent 2 hours.

Figure 2 demonstrates that treatment with pH 10- 11 slightly increased the binding of rabbit IgG to WBP during the first 90 min by 10%. Then the IgG binding decreased to the initial value after 120 min of treatment. After three hours, recognition of these proteins by IgG was slightly reduced by 6% compared to untreated samples. The acidic pH decreased the binding of IgG to WBP by 21.8% during 90 min. The reduction remained stable during later hours without statistically significant modification.

Pepsin hydrolysis in an acid environment greatly impaired the binding of IgG to WBP which was reduced by 46.6% in the first 30 min of treatment, and remained relatively constant thereafter.

Immunoreactivity of Treated WBP with Human IgE

In this section, the change in the recognition of treated WBP (by heating, pH, and pepsin) by human IgE was determined. To do this, human sera were used

Effect of Enzymatic Treatments on Sensitivity to White Bean Allergens

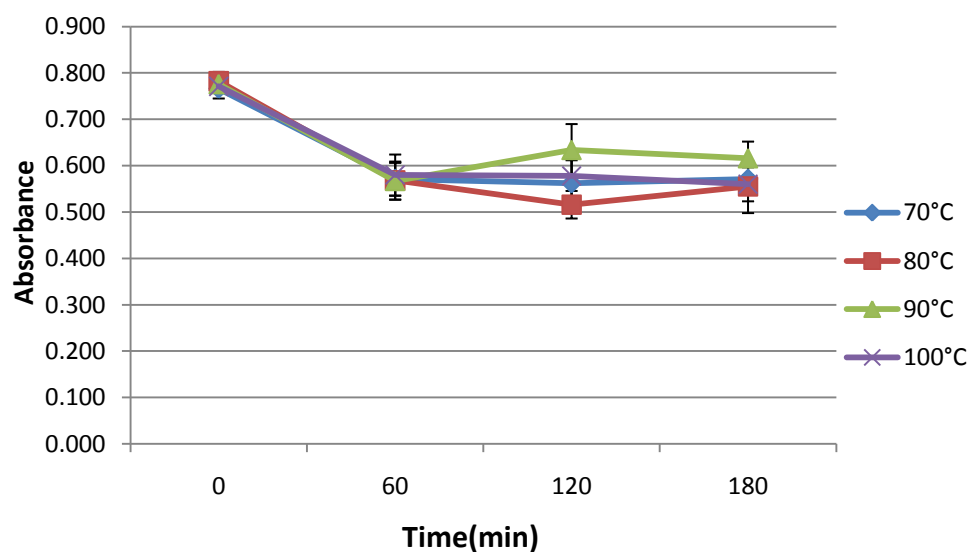


Figure 1. Effect of heat treatment on the recognition of WBP by rabbit IgG. The effect of temperature was studied in N=3 experiments using rabbit IgG anti-native WBP. The binding of IgG was evaluated by ELISA for determining the response of IgG binding to WBP, heated at different temperatures over time. Compared to control value (at t=0 min), all values are statistically significant with at least $p < 0.01$.

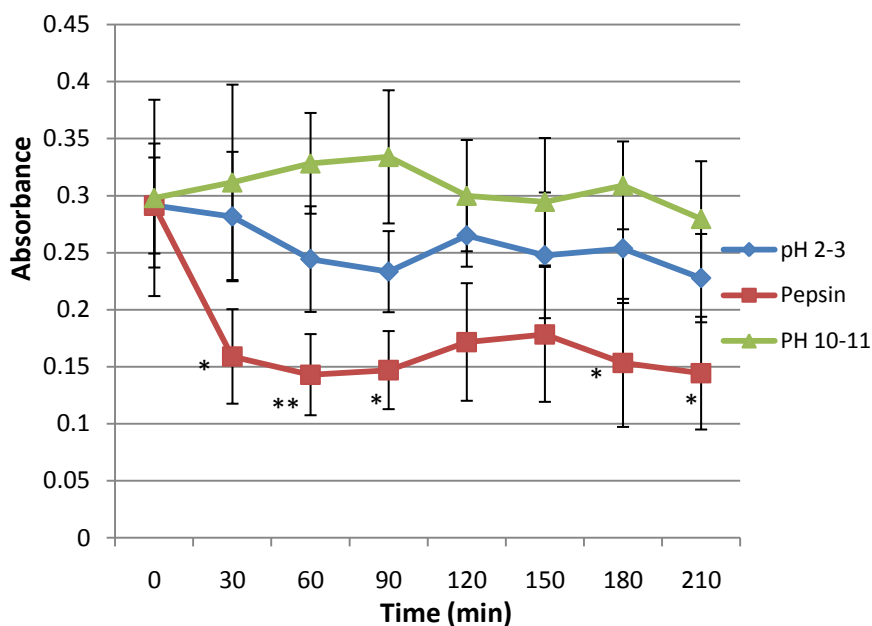


Figure 2. Effect of acid (pH 2-3), alkali (pH 10-11) treatment and enzymatic hydrolysis on the recognition of WBP by rabbit IgG. The effect of these treatments were studied in N=3 experiments ($*p < 0.05$; $**p < 0.01$) using rabbit IgG anti-native WBP. The binding of IgG was evaluated by ELISA for determining the response of IgG binding to WBP hydrolyzed in alkali, acid or by pepsin at 37°C over time.

Table 1. Specific IgE measured in patients.

Topics	Children				Adults			Average
Patients	120	2	43	50	17	92	28	
Sex	M	F	F	F	F	F	M	
Age in years	5	9	10	10	18	19	49	17.14
Specific IgE (in IU/ml)	16.5	38.5	68.1	64.2	30.1	16.9	22.8	36.7

M: Male, F: Female

from seven patients with bean IgE levels higher than 16 IU/ml. Patients were composed of 4 children (one boy and 3 girls) with age ranging from 5 to 10 years, and three adults (one man and two women). Specific IgE ranged from 16.5 to 68.1 IU/ml (Table 1).

When WBP were heated at 80°C for 60 min, the results reported in Figure 3 showed an average reduction in IgE recognition of 68% for all patients, ranging from 37 to 94%. All of the patients indicated a reduction greater than 36%, while 71% of these patients showed a decrease greater than 67% of the recognition by IgE.

Under pepsin hydrolysis, the same average reduction of 68% was obtained for all patients with a reduction greater than 31% for all patients.

When the two treatments were combined (heating and pepsin), seven patients showed a 62% average

reduction of the recognition of WBP. This reduction varied from 19% to 100% with a different panel of responses. For example, a total reduction for patient N°28 who was an adult (man, 49 years) was observed, where patient N°120 (boy, 5 years) indicated a very slight reduction of 19%. These results under combination of treatments; indicated also that 5 out of 7 patients showed a reduction less than that observed with heating or pepsin alone (Figure 3).

Electrophoretic Analysis

The protein composition of various extracts of beans (native, heated and/or treated with pepsin) were compared by polyacrylamide gel electrophoresis under denaturing and non-denaturing conditions (Figure 4)..

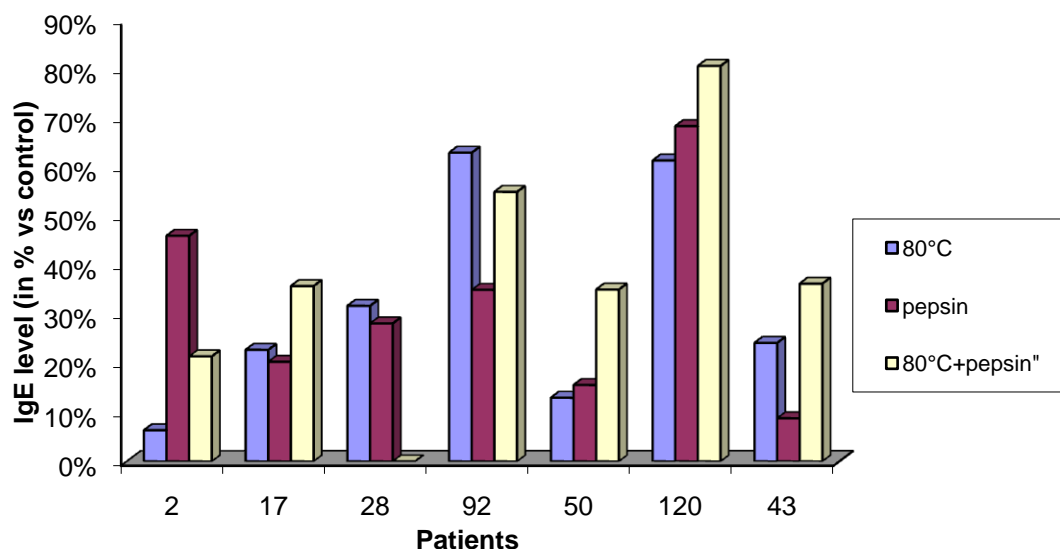


Figure 3. Effect of heating and enzymatic hydrolysis on the recognition of WBP by human IgE. Figure represents the IgE level in % compared to data obtained in control (No treated WBP). Values were obtained in the three different conditions, heating, pepsin hydrolysis and combination of these treatments. In x-axis, numbers represent the laboratory identification number of patient's sera.

Effect of Enzymatic Treatments on Sensitivity to White Bean Allergens

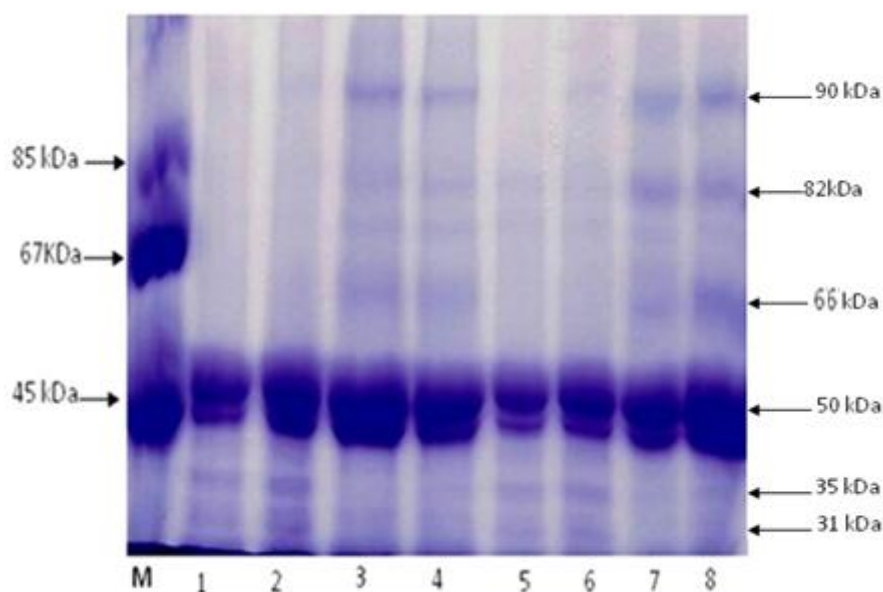


Figure 4. Electrophoresis profile of WBP native (7, 8); processed by enzymatic hydrolysis (6, 5); processed by temperature (4, 3); processed by temperature and enzymatic hydrolysis (1,2). In denaturing conditions (2, 4, 6, 8) and undenaturing conditions (1, 3, 5, 7). The markers were added in the well.

Before treatment, the native bean extracts under denaturing conditions showed an electrophoretic profile consisting of six protein bands of molecular weights ranging from 30 to 90 kDa with a major band of about 50 kDa. The same pattern was observed under non-denaturing conditions, except that there was a disappearance of protein bands below 50 kDa.

However, after heat treatment, there was a slight attenuation of all protein bands under denaturing and non-denaturing conditions except the protein bands of 50 kDa and 90kDa.

For the bean extracts treated with pepsin or pepsin and heat, the proteins above 50 kDa disappeared with an apparition of proteins with lower molecular weight of about 31 and 35 kDa in denaturing and non-denaturing conditions.

DISCUSSION

In the present work, we were interested in studying Moroccan population's sensitivity to white bean proteins in two regions (Fez and Casablanca) and we investigated the effect of thermal processes and enzymatic digestion on their antigenicity. Indeed, a sera bank of 146 patients with atopic hypersensitivity was established in order to assess the specific IgE levels

directed against bean proteins, in a second part we have evaluated recognition of native and treated bean proteins by rabbit IgG or human IgE.

The results showed that 51% of children and 39% of adults showed positive specific IgE values. Of these, 20% of children and 40% of adults showed a rate higher than 20 IU/ml. The IgE levels showed a strong sensitivity of the studied population to bean proteins which was explained by daily consumption of white beans by Moroccans. From Food Agriculture Organization (FAO) data; in year 2000, the average consumption of dry beans in the world was estimated to be 2.2 kg per capita per year: Africa 2.2 kg, Asia 1.3 kg, Europe 0.7 kg, Morocco 0.5 Kg.

In the world, several cases of bean sensitizations were reported in different countries.^{14, 16, 22} In Spain (nearby country), sensitivity to legumes is the fifth most prevalent food allergy among children's population.⁵ In India, sensitivity observed to kidney beans was 22% among patients' allergic legumes and about 90% of the patients studies showed specific IgE to bean proteins.²²

The results of WBP heat treatment showed a significant reduction in the recognition of these antigens by rabbit IgG, with 25% of reduction at all temperatures studied. Under the same condition,

heating highly reduced WBP recognition by human IgE. All patients indicated a reduction greater than 36%, while 71% of these persons showed a decrease greater than 67% of the recognition by IgE. These findings could be explained by a fragmentation of a part of proteins as suggested by the protein band attenuation observed by electrophoresis. The higher decrease observed by IgE compared to IgG, suggests that some antigens recognized by IgE in the studied population were conformational labile sites where majority of antigens recognized by rabbit IgG were sequential sites.

Dry beans, in their protein composition, contain a large part of phytohemagglutinin (PHA) and non-PHA proteins.²³⁻²⁵ These later proteins were showed to be sensitive to heating and cooking.²⁶⁻³⁰ The PHA was observed to be the most allergenic protein.^{16,22} By electrophoresis, we observed six protein bands of molecular weights between 30 and 90 kDa with a major band of about 50 kDa. After heat treatment, there was a slight attenuation of protein bands between 90 and 50 kDa except for proteins of 50 kDa and 90 kDa. These indicated that proteins attenuated by heating were probably sites recognized by human IgE. These findings are in accordance with data obtained by Kasera et al.²²

When enzymatic processing by pepsin was studied, a decrease in the IgE recognition of WBP in the patients studied was observed with an average reduction of 68% for all patients and greater than 31%. The same decrease was obtained with rabbit IgG anti-native WBP. This means that pepsin cleaved at the sites of WBP allergenicity, which can result in the elimination of some epitopes. This result is comparable to that observed in peanuts, where digestibility decreased allergenicity of Arah1 by pepsin degradation.³¹

After the combination of the two treatments (heating and pepsin), an average reduction of WBP recognition of approximately 62% was obtained. This reduction under combination of treatments was less than that observed with heating or pepsin alone for 5 of 7 patients studied. It was observed previously that digestibility of bean proteins was enhanced after the beans were roasted³² or cooked³³. This indicates that pepsin cleavage was more effective after cooking. Our results can be explained by the apparition of new allergenic sites due to the action of pepsin on heated WBP.

When WBP was hydrolyzed by pepsin alone or after protein heating, electrophoresis analysis showed that bands above 50 kDa were lost along with an appearance of bands of 35 and 31 kDa. This is supposed that such peptides generated by the action of pepsin on heat denatured bean proteins, expose new epitopes responsible for the increased allergenicity after combining the two treatments.

In conclusion, these results demonstrate that the Moroccan population is very sensitive to WBP and this sensitivity could be reduced by heat treatment or enzymatic hydrolysis. The combination of both treatments reduced immunoreactivity slightly by discovering new allergenic sites for several analyzed patients.

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Effect of Enzymatic Treatments on Sensitivity to White Bean Allergens

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