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Effects of Extrusion, Boiling, Autoclaving, and Microwave Heating on Lupine Allergenicity

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Lupine flour has been reported as a causative agent of allergic reactions. However, the allergenicity of lupine after thermal processing is not well-known. For this purpose, the allergenic characteristics of lupine seeds after boiling (up to 60 min), autoclaving (121 °C, 1.18 atm, up to 20 min and 138 °C, 2.56 atm, up to 30 min), microwave heating (30 min), and extrusion cooking were studied. The IgE-binding capacity was analyzed by IgE-immunoblotting and CAP inhibition using a serum pool from 23 patients with lupine-specific IgE. Skin testing was carried out in four patients. An important reduction in allergenicity after autoclaving at 138 °C for 20 min was observed. IgE antibodies from two individual sera recognized bands at 23 and 29 kDa in autoclaved samples at 138 °C for 20 min. Autoclaving for 30 min abolished the IgE binding to these two components. A previously undetected band at 70 kDa was recognized by an individual serum. Therefore, prolonged autoclaving might have an important effect on the allergenicity of lupine with the majority of patients lacking IgE reactivity to these processed samples.

KEYWORDS: Lupine; autoclave; food hypersensitivity; thermal processing; legumes; neoallergen

INTRODUCTION

Sweet white lupine (SWL) (*Lupinus albus*), a widely cultivated legume, has been shown to increase the protein and fiber crops in conjunction with durum wheat in specialty pasta and to be an excellent source of white fiber, as an additive to breads and cereals (*1*−*3*). Moreover, recent studies indicate that lupine may provide a useful alternative for individuals wishing to substitute animal with plant proteins for cardiovascular disease prevention (*4*). At present, there is a developing specialty human food market for lupine in the form of lupine flour, lupine pasta, and hulls for dietary fiber. Allergic reactions to lupine-fortified pasta were first reported by Hefle et al. in 1994 (*5*). The ingestion of lupine seed flour has since been a well-established cause of IgE-mediated allergic reactions, especially in patients sensitized to peanut (*6*−*9*). Moneret-Vautrin et al. (*10*) demonstrated the existence of immunologic cross-reactivity between lupine flour and peanut, which was clinically relevant in approximately one-third of 24 patients with peanut allergy. Thus, the risk of lupine allergy in patients with peanut allergy seems to be higher than cross-allergy to other legumes. Furthermore, our previous studies have shown that the inhalation of lupine flour could be an important cause of allergic sensitization in exposed workers and might give rise to occupational asthma and food allergy (*11*).

Lupine seed proteins have proved to be an interesting model to study protein thermal conformational stability under different pH conditions from both biochemical and technofunctional points of view (*12*, *13*). Moreover, it has been suggested that modifications in allergenicity caused by thermal treatments could provide a better understanding of the risk of introducing lupine into human daily food intake (*10*). Therefore, the present study was specifically designed to evaluate the effect of boiling, autoclaving, microwave heating, and extrusion cooking on the allergenic characteristics of lupine.

MATERIALS AND METHODS

Patient Sera. Serum samples were obtained from 23 patients with positive specific serum IgE to lupine (*Lupinus albus*), as quantified by using the CAP-FEIA system (Pharmacia Diagnostic, Uppsala, Sweden). All individual sera showed specific IgE levels ranging between 0.7 and 87.1 kilounits/L (median = 4.1 kilounits/L), and the serum pool comprising the 23 sera had 6.9 kilounits/L. The control serum showed IgE specific to almond (3.35 kilounits/L), sunflower seed (0.47 kilounits/L), chickpea (0.52 kilounits/L), and <0.35 kilounits/L to lupine seed.

Plant Material, Heat Treatments, and Protein Extracts. SWL seeds (*L. albus* cv. Multolupa) obtained from the Servicio de Investigación y Desarrollo Agrario (Badajoz, Spain) were subjected to different treatments, including boiling, microwave heating, autoclaving, and extrusion cooking. Seeds (1:10 w/v) were boiled in water and in Tris buffer (0.05 M Tris-HCl, pH 8.0, plus 0.5 M NaCl) at 100 °C for 15,

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30, and 60 min. Using a tabletop autoclave (CertoClav Multicontrol IPX4, Traun, Austria), lupine seeds (1:5 w/v) were treated at 121 °C (1.18 atm) for 5 and 20 min and at 138 °C (2.56 atm) for 5, 20, and 30 min. Microwave heating was performed in a microwave oven (Whirpool TC 2280, Nörrköpping, Sweden) for 30 min at 750 and 900 W in water (1:10 w/v). For extrusion cooking, the lupine flour was mixed with corn flour (67% lupine and 33% corn) and treated at the Departamento de Ciencias del Medio Natural from the Escuela Técnica Superior de Ingenieros Agrónomos (Universidad Pública de Navarra, Pamplona, Spain) in a Clextral X-5 BC-45 (Firminy, France), corotating twin-screw extruder model F-42100 (Firminy, France) operated at 135 °C, 250 rpm screw speed, and a constant moisture content (7%). The extrudates were allowed to cool to room temperature and were reground to pass through a 0.5 mm sieve.

Raw and thermally processed seeds were milled to pass through a 1 mm sieve (Tecator, Cyclotec 1093, Höganäs, Sweden), and the resulting meal was defatted with *n*-hexane (34 mL/g of flour) for 4 h, shaken, and air-dried after filtration of the *n*-hexane (*14*). Defatted flour was extracted twice in a solution of 0.05 M Tris-HCl, pH 8.0, plus 0.5 M NaCl at a 1:10 w/v ratio for 1 h at 4 °C by stirring. The extract was clarified by centrifugation at 27000*g* for 20 min at 4 °C, and the supernatants were dialyzed against H_2O for 48 h at 4 °C using a dialysis membrane with a cutoff of 3.5 kDa and freeze-dried. The protein content of each sample was measured according to the Bradford dye-binding assay (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA; Sigma, St. Louis, MO) as a standard.

Protein Electrophoresis, Immunoblotting, and Immunoblotting Inhibition. Denaturing protein electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (*15*). Samples (50 μg per well) were mixed 3:1 with loading buffer (8% SDS, 8 mM EDTA, 40% glycerol, 1 M β-mercaptoethanol, and 0.01% bromphenol blue in 0.25 M Tris-HCl, pH 7.5) heated at 100 °C for 10 min, electrophoresed in 12% analytical SDS−polyacrylamide gels employing a Mini-Protean III apparatus (Bio-Rad), and either stained with Coomassie Brilliant Blue R-250 or transferred to poly(vinylidene difluoride) (PVDF) membranes (Bio-Rad). For immunoblot, proteins were electrophoretically transferred from the gels to PVDF by applying a constant current of 250 mA during 2 h at room temperature, essentially according to the method of Towbin et al. (*16*). A piece of membrane was stained with amido-black to verify protein transfer. Blots were blocked in phosphate-buffered saline plus 0.1% Tween 20 containing 1% fatfree milk powder for 1 h at room temperature and then incubated overnight at room temperature with individual and pooled sera (1:4 dilution). In the case of inhibition experiments, lupine extracts (1 mg/mL) were added to the patients' sera 3 h before application to the blot membrane. A polyclonal goat anti-human IgE conjugated with alkaline phosphatase was used as second antibody (diluted 1:500) (Caltag, Burlingame, CA). Finally, immunoreactive bands were visualized using the alkaline phosphatase conjugate substrate kit (Bio-Rad). Nonspecific binding of the anti-IgE antibody conjugate was measured in a similar blotting procedure, omitting the incubation step with patient sera.

Coomassie-stained gels and immunostained membranes were scanned using a GS 800 densitometer (Bio-Rad), and the generated files were analyzed with Quantity One software (Bio-Rad) using the low-range prestained SDS-PAGE protein mixture (Bio-Rad) as standard.

CAP Inhibition Assays. The serum was incubated with the same volume of progressive dilutions (0.001−1 mg/mL) of each inhibitor protein solution in PBS buffer for 14 h at 4 °C with agitation. After incubation, the samples were centrifuged, lupine commercial ImmunoCAP was then added, and specific IgE levels were assessed, according to the CAP System procedure (Pharmacia Diagnostic, Uppsala, Sweden). BSA was used as negative control. The assay was performed in duplicate samples, and each one was tested in duplicate.

Skin Prick Tests (SPT). SPT were carried out on the volar side of the forearm according to standard methods (*17*). A positive SPT result was defined as a wheal 3 mm or greater in diameter in comparison with the negative control. Positive and negative controls for skin testing were histamine dihydrochloride (10 mg/mL) and PBS buffer, respectively.

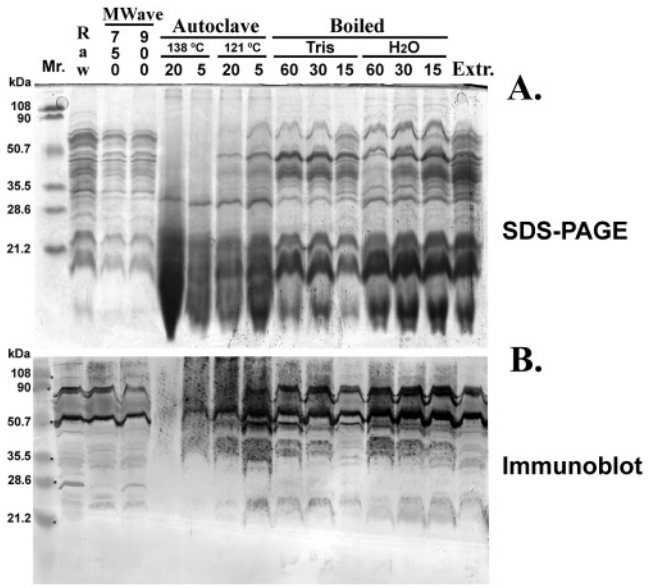

Figure 1. SDS-PAGE (**A**) and IgE-immunoblotting (**B**) analysis of raw (lane 1), microwave heated (lanes 2 and 3), autoclaved (lanes 4–7), boiled (lanes 8–13), and extrusion cooked (lane 14) processed SWL seed extracts. The immunoblotting was carried out using a serum pool from 23 patients with specific IgE to raw SWL, determined by a CAP-FEIA system. Mr., molecular weight markers; Mwave, microwave heating at 750 and 900 W for 30 min; autoclave, autoclaving at 138 or 121 °C for 5 and 20 min; boiled, boiling in water or Tris buffer for 15, 30, and 60 min; Extr., extrusion.

Table 1. CAP Inhibition Assays of Raw Lupine by Raw, Microwave Heating, Autoclaving, Boiling, and Extrusion Cooking Extracts[a]

inhibitor	% inhibition ± SD
microwave	
750 W	92.0 ± 0.2
900 W	88.8 ± 1.2
autoclave 138 °C	
20 min	58.0 ± 0.5
5 min	70.0 ± 2.3
autoclave 121 °C	
20 min	82.4 ± 0.8
5 min	84.8 ± 0.6
boiled in Tris	
60 min	79.4 ± 0.2
30 min	89.7 ± 0.7
15 min	87.9 ± 0.2
boiled in H_2O	
60 min	72.9 ± 0.1
30 min	90.6 ± 0.3
15 min	91.2 ± 0.6
extrusion	93.8 ± 0.1
raw	93.7 ± 0.3
BSA	0.01 ± 0.02

[a] A pool of sera from 23 patients with raw lupine-specific IgE was preincubated with each inhibitor solution at a 1 mg/mL final concentration.

RESULTS

Microwave-heated, autoclaved, boiled, and extrusion-cooked SWL extracts were compared with raw SWL by SDS-PAGE analysis (**Figure 1A**). The protein profiles from processed samples by microwave heating, boiling, and extrusion cooking remained essentially unchanged, whereas some protein bands seemed to be clearly reduced in autoclaved lupine extracts, particularly at 138 °C. Immunoblot experiments (**Figure 1B**) using the serum pool revealed that IgE-binding components are generally heat stable, and important reductions in allergenicity

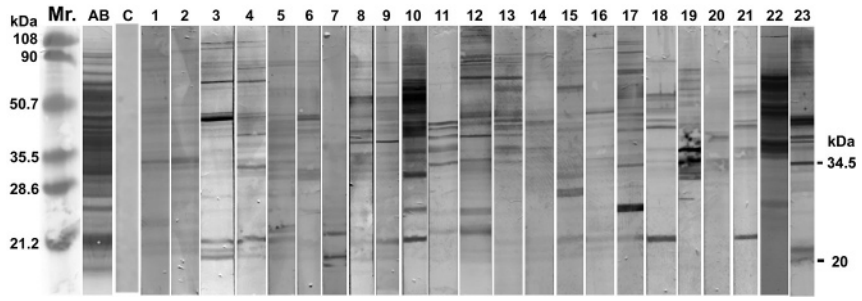

Figure 2. IgE antibody reactivity to raw SWL (*L. albus*) allergens by immunoblotting. Lupine proteins were resolved by means of SDS-PAGE and transferred to PDVF membranes. Five-millimeter strips from each blot were tested for reactivities to serum IgE from 23 patients sensitized to raw lupine-specific IgE. Lane Mr. is the molecular weight standard; lane AB represents staining for proteins by Amido Black; lane C represents a negative control of a serum from a patient without specific IgE to lupine; lanes 1–23 represent sera from patients 1–23, respectively. Arrows indicate the location of major allergens.

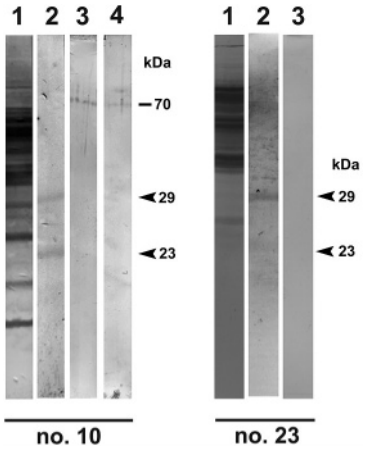

Figure 3. IgE antibody reactivity to raw (lanes 1) and autoclaved at 138 °C for 20 min (lanes 2) and 30 min (lanes 3) SWL extracts by SDS-PAGE immunoblotting using sera 10 and 23. Lane 4 represents Immunoblot inhibition assay of autoclaved at 138 °C for 30 min SWL extract by raw SWL extract using the serum number 10. The serum was preincubated with inhibitor solution at 1 mg/mL final concentration. Arrows indicate the molecular weights.

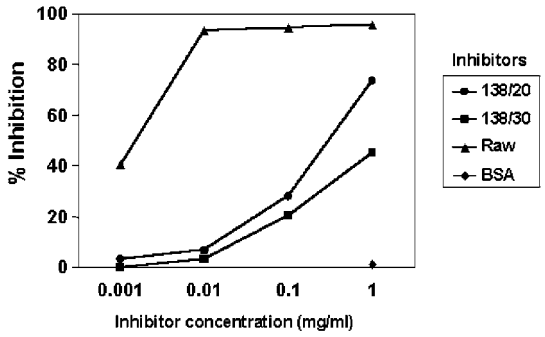

Figure 4. CAP inhibition assays of raw SWL by autoclaved (138 °C) for 20 (●) and 30 (■) min SWL extracts using serum 10. No relevant inhibition was obtained by using BSA as a negative control.

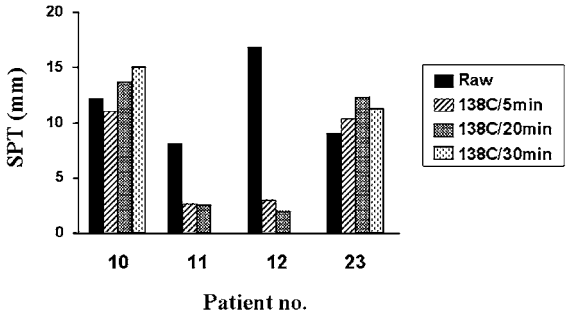

Figure 5. Histogram representation of skin prick test results with raw and autoclaved (138 °C) SWL extracts and histamine in patients who reacted (no. 10 and 23) or not (no. 11 and 12) to autoclaved SWL extracts by IgE immunoblot. Bars represent the mean value of orthogonal diameters of wheal in mm.

of certain protein bands are observed only under autoclaving at 138 °C for 20 min. Boiling of lupine seeds in water or in Tris buffer showed a similar pattern of allergenic proteins and IgE-binding intensities except for a 20 kDa band, which was detected only in the water-boiled samples. In parallel with the findings from immunoblot experiments, data from direct inhibition CAP-FEIA assays with a commercial raw SWL extract as the solid phase (**Table 1**) indicated a relevant decrease in allergenicity at certain extremes of prolonged autoclaving (138 °C for 20 min).

IgE antibody reactivity to raw and autoclaved SWL was further screened using the individual sera. IgE immunoblots of raw SWL extract probed with the individual sera of 23 patients are shown in **Figure 2**. A complex pattern of bands from 19 to 108 kDa was detected, with proteins at 20 and 34.5 kDa recognized by 12 of the 23 (52%) individual sera. Only 2 (no. 10 and no. 23) of 16 (12.5%) sera tested reacted to autoclaved samples at 138 °C for 20 min. IgE antibody from these sera recognized two components at 23 and 29 kDa (**Figure 3**, lanes 2). Autoclaving of SWL at 138 °C for 30 min abolished the IgE antibody reactivity from these sera to the previously detected components (**Figure 3**, lanes 3). However, serum 10 recognized a previously undetected band at 70 kDa (**Figure 3**, lane 3), which remained reactive when serum was preincubated with raw SWL extract (**Figure 3**, lane 4). Dose-dependent CAP inhibition assays with a commercial raw SWL extract rendered progressive inhibition values up to 73% and 45 for the autoclaved samples (138 °C) for 20 and 30 min preincubated with serum 10, respectively (**Figure 4**).

Skin testing with raw and autoclaved (138 °C for 5, 20, and 30 min) SWL extracts was carried out on patients whose sera reacted, or not, to autoclaved SWL extracts in IgE immunoblot experiments. Likewise, autoclaved extracts at 138 °C elicited manifest skin reactivity only in patients 10 and 23 (**Figure 5**).

DISCUSSION

Our study demonstrates that processing procedures, such as microwave heating, boiling, and extrusion cooking, produce minimum changes on the SDS-PAGE protein profile and IgE recognition of lupine allergens. To date, there are only a few

systematic investigations of thermal-processing parameters, which potentially influence the allergenicity of legume allergens (*18*−*22*). Beyer et al. (*22*) demonstrated that the methods of frying (120 °C, 5−10 min) and boiling (100 °C, 20 min) peanuts reduced IgE-binding intensity to the major peanut allergens Ara h 1, Ara h 2, and Ara h 3 compared with dry roasting (170 °C, 20 min). Modifications in allergenicity caused by the method of peanut cooking have been invoked to play a role in the difference in the prevalence of peanut allergy in various countries around the world. Burks et al. (*20*) determined no significant decrease of IgE binding in RAST inhibition after heating (100 °C, up to 60 min) of soybean protein extracts. However, boiling for 120 min and microwave heating (700 W, 25 min) of soybeans seems to decrease allergenicity, as only half of soybean-allergic patients were shown to have detectable specific IgE against heated soybean protein (*18*). Conversely, boiling (100 °C, 15 min) of lentil seeds produces substantial changes in the electrophoretic pattern (*23*), with a strong increase in allergenicity as demonstrated by ELISA inhibition experiments (*24*). Therefore, thermal-processing procedures at various temperatures and conditions could have unpredictable effects on the allergenic activity and support the necessity of systematic investigations of new ingredients to allow more informed and accurate risk analysis.

In contrast with some model studies, which imitate ordinary thermal-processing methods (mainly, boiling and conventional dry-heating), we investigated the effect on lupine allergenicity of harsh food-processing conditions, such as extrusion and pressure cooking in an autoclave. Our results suggest that only autoclaving at 138 °C produces an important effect on the integrity and structure of lupine proteins, which corresponds to a relevant decrease on the overall allergenicity. After autoclaving for 20 min, maximum CAP inhibition was ∼58% as compared to raw lupine extract and only 2 of 16 sera from lupine-sensitive patients showed IgE binding in SDS immunoblot. The effect of autoclaving on food allergenicity has been hardly ever investigated. Almost three decades ago, Malley et al. (*25*) demonstrated that the albumin fraction from green peas retains its allergenicity upon heating at 60 °C for 30 min or boiling at 100 °C for 5 min, but becomes partially inactivated by autoclaving at 120 °C for 15 min. Recently, Venkatachalam et al. (*26*) showed that the allergenicity of almond proteins is maintained after autoclaving at 121 °C, up to 30 min, but the effect of thermal treatments at 138 °C were not explored.

In the present study the allergenic potency was considerably lower, or even abolished, after autoclaving of lupine for 30 min in comparison with preparations autoclaved for 20 min. These differences in allergenicity were observed in parallel with notable modifications in the IgE recognition profile of lupine allergens in SDS immunoblot. After autoclaving for 20 min, only two bands of 23 and 29 kDa conserved the IgE-binding capacity. Interestingly, autoclaving at 138 °C for 30 min abolished the IgE recognition of these components, but generated a previously undetected IgE-binding component (70 kDa). Neoallergens have been described in other vegetal species such as soybean (*27*) and pecan nut (*28*) after storage and heat treatment. The consecutive appearance of different allergenic components illustrates the complexity of chemical modifications that occur through thermal processing and its deep influence on allergenicity. These modifications include the generation of Maillard products and other covalent modifications of proteins, such as reactions with oxidized lipids, direct oxidation through reactive oxygen intermediates, disulfide bond scrambling, and deamination of asparagines (*29*, *30*).

In conclusion, although further studies are needed to assess the clinical relevance of our findings, autoclaving seems to be a promising method of decreasing the allergenicity of legume seeds

ABBREVIATIONS USED

BSA, bovine serum albumin; FEIA, fluorescent enzyme immunoassay; PVDF, p oly(vinylidene difluoride); SPT, skin prick test; SWL, sweet white lupine (*Lupinus albus*).

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