RESEARCH ARTICLE

Influence of thermal processing on IgE reactivity to lentil and chickpea proteins

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In the last years, legume proteins are gaining importance as food ingredients because of their nutraceutical properties. However, legumes are also considered relevant in the development of food allergies through ingestion. Peanuts and soybeans are important food allergens in Western countries, while lentil and chickpea allergy are more relevant in the Mediterranean area. Information about the effects of thermal-processing procedures at various temperatures and conditions is scarce; therefore, the effect of these procedures on legume allergenic properties is not defined so far. The SDS-PAGE and IgE-immunoblotting patterns of chickpeas and lentils were analyzed before and after boiling (up to 60 min) and autoclaving (1.2 and 2.6 atm, up to 30 min). The results indicated that some of these treatments reduce IgE binding to lentil and chickpea, the most important being harsh autoclaving. However, several extremely resistant immunoreactive proteins still remained in these legumes even after this extreme treatment.

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1 Introduction

Legumes are increasingly being regarded as beneficial food ingredients. In fact, they are recommended as staple food by health organizations [1], and dieticians are nowadays tending to encourage their consumption in counseling [2]. Lentils and chickpeas have been reported as a cause of IgE-mediated hypersensitivity reactions, particularly in pediatric patients [3–5]. The frequency of allergy to lentil and chickpea in the Spanish population has been estimated around 20% of children with food allergy [6]. Some subjects allergic to these legumes on ingestion also report symptoms when they inhale vapors from cooking lentils or chickpeas [7–9].

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Abbreviations: DIC, instantaneous controlled pressure-drop; **PVDF,** polyvinylidene difluoride

Studies investigating the allergenicity of lentils and chickpeas are scarce. So far, no chickpea allergen has been identified but several IgE-binding bands with molecular weights between 10 and 70 kDa have been detected by immunoblotting [5, 9, 10]. A major lentil allergen Len c 1 has been isolated and identified as a 48 kDa vicilin; its processing fragments, corresponding to subunits 12–16 and 26 kDa are also relevant lentil IgE-binding proteins [11, 12].

Heating promotes protein denaturation, aggregation, and structure disruption and therefore has a potential to modify allergenic properties of proteins [13]. The molecular basis of changes in allergenic activity is the inactivation or destruction of epitope structures, the formation of new epitopes, or an enhanced access to cryptic epitopes by denaturation of the native allergen [13]. Plant protein allergenicity may be variably affected by thermal processing, *i.e.* increasing or decreasing IgE immunoreactivity. Thus, the overall effect of this procedure on a complex food allergen cannot be

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predicted [14–18]. It has been reported that lentil and chickpea allergens are heat stable after boiling [10, 11, 19]. Our previous studies have observed that boiling in autoclave reduced lupine allergenicity drastically, even abolishing IgE binding [20]. Recently, the high thermal resistance of the major lupine allergens has been established using instantaneous controlled pressure-drop (DIC) treatment, a procedure that combines heat and steam pressure as autoclaving [21].

The aim of this work was to assess changes in the IgE-binding capacity of lentil and chickpea proteins by means of thermal-processing techniques such as boiling and autoclaving.

2 Materials and methods

2.1 Sera

Three different sera groups were employed: (i) 25 individual sera with positive specific IgE to lentil (*Lens culinaris*) (range: 0.53–30.7 kU/L) as quantified by using the CAP-FEIA (fluorescent enzyme immunoassay) system (Pharmacia Diagnostic, Uppsala, Sweden). (ii) Twenty-four individual sera with positive specific IgE to chickpea (*Cicer arietinum*) (range: 0.45–29.6 kU/L). (iii) A serum pool from a different source having specific IgE levels to lentil and chickpea of 31.3 and 29.0 kU/L, respectively. A serum from a patient with specific IgE to *Anisakis* ssp. (9.09 kU/L), specific IgE <0.35 kU/L to lentil and chickpea, and a total serum IgE value of 53.4 kU/L was used as a negative control.

2.2 Plant material, heat treatments, and protein extracts

Mature lentil seeds (*L. culinaris* var. Guareña) obtained from the Servicio de Investigación y Tecnología Agraria (Valladolid, Spain) and mature chickpea seeds (*C. arietinum* var. Athenas) obtained from the IFAPA (Córdoba, Spain) were subject to different treatments, including boiling and autoclaving. Seeds (1:10 w/v) were boiled in water at 100°C for 15, 30, and 60 min. Using a table top autoclave (CertoClav model IPX4, Austria), legume seeds (1:5 w/v) were treated at 1.18 and 2.56 atm for 5, 15, and 30 min in the case of lentils and 15 and 30 min in the case of chickpeas.

Raw and thermal-processed seeds were milled to pass through a 1 mm sieve (Tecator, Cyclotec 1093, Sweden) and the resulting meal was defatted with *n*-hexane (34 mL/g of flour) for 4 h with agitation and air-dried after filtration of the *n*-hexane. Defatted flour was extracted with 0.1 M PBS buffer pH 7.4 plus 0.15 M NaCl at a 1:10 w/v ratio during 1 h at 4°C with stirring. The extract was clarified by centrifugation at $27\,000 \times g$ for 30 min at 4°C, and the supernatants were dialyzed against distilled H_2O during 24 h at 4°C using dialysis membrane (Spectra/Por, Serva, Heidelberg, Germany) with a cut-off of 3.5 kDa and then freeze-dried. The residue obtained after extraction and the cooking water were freeze-dried for analysis. The soluble protein content of each sample was measured by the Bradford dye-binding assay (Bio-Rad, Hercules, CA, USA) using BSA (Sigma, St. Louis, MO, USA) as a standard. The total N was determined using the Kjeldahl procedure [22]. The total crude protein content was calculated as $N \times 5.45$ [23].

2.3 Protein electrophoresis and immunoblotting

Denaturing protein electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [24]. Samples were mixed with XT Sample Loading Buffer (Bio-Rad) and XT Reducing Agent (Bio-Rad), heated to 90°C for 10 min, and electrophoresed in 12% Bis-Tris Criterion XT Precast Gel (Bio-Rad). Proteins (16 μg protein/lane) were visualized with CBB R250 staining. IgE immunoblotting analysis was performed by electrophoretic transfer to polyvinylidene difluoride (PVDF) at 250 mA during 100 min at room temperature [25]. After blocking with 5% BSA w/v in PBS buffer, membranes were incubated overnight with the serum pool or individual sera (1:10 dilution), washed, and then treated with mouse anti-human IgE mAb HE-2 ascitic fluid (1:3000 dilution for 2 h) [26]. After washing, a rabbit anti-mouse IgG peroxidase-conjugated antibody (1:5000 dilution for 1 h; DAKO, Glostrup, Denmark) was added. Detection of IgE-binding components was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Amersham Biosciences, Little Chalfont, UK).

Coomassie-stained gels and membranes were scanned and the molecular weight of the bands was assessed using Quantity One software (Bio-Rad) and the low-range SDS-PAGE protein mixture (Sigma) as standard.

2.4 Skin prick tests

Skin testing was carried out after *in vitro* experiments were completed. For this purpose, 12 additional patients sensitized to legumes were asked to undergo skin testing with raw and thermal-processed extracts. After obtaining the informed consent, approved by the Ethic Committee (Permission No. 0312150129) skin prick tests (1:10 w/v) were performed in duplicate on the volar side of the forearm according to standard methods [27]. A mean wheal diameter of 3 mm or greater (15 min after puncture) compared with that produced by the negative control was considered a positive response. Positive and negative controls for skin testing were histamine dihydrochloride (10 mg/mL) and saline solutions, respectively.

3 Results

Thermal processing can make that some seed proteins be un-extractable and left in the residue and some dissolve in the cooking medium. Therefore, the protein content, SDS-PAGE, and IgE-immunoblotting have been analyzed in defatted legume flour, extract, residue, and cooking water of lentil and chickpea before and after heat treatments (data not shown). The results supported that more protein remained in the residue as un-extractable material upon processing than in raw samples and part of extractable protein were solubilized into the cooking water. However, there was not substantially different IgE-immunoreactivity profile among these fractions (flour, extract, residue, and water); therefore, the IgE-binding capacity of the PBS extract could be considered as representative of the overall immunoreactivity of these foods.

3.1 Effects of heating on lentil allergens

Figure 1 shows the SDS-PAGE protein patterns of raw and thermal-processed lentil extracts. All these extracts were analyzed by IgE-immunoblot using the serum pool (Fig. 2). SDS-PAGE and IgE-immunoblot band patterns are similar in raw and 15-min boiled lentil showing multiple allergenic proteins. After boiling for 30 min, a putative Len c 1, the major lentil allergen, is eliminated, but multiple immunoreactive proteins are still present. The autoclave process at harsh conditions (2.6 atm) induces major changes in lentil immunoreactivity (lanes 8–10). The number and intensity of bands are reduced after 15 min autoclaving (2.6 atm) with several heat-stable allergenic proteins still being present. A few extremely resistant immunoreactive bands (16, 18, 21, 30, and 43 kDa) are detected at the highest pressure and treatment time applied (lane 10).

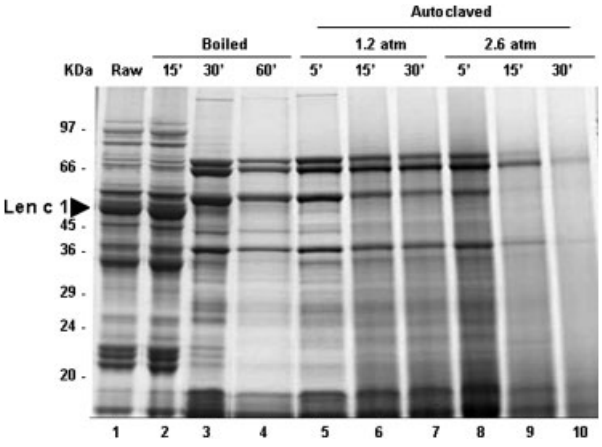

Figure 1. SDS-PAGE patterns of protein extract from raw (lane 1) and processed lentil (lanes 2–10) samples. Arrow indicates the position of the major lentil allergen.

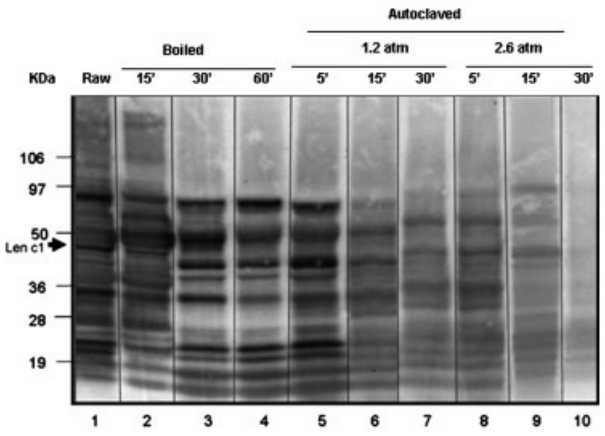

Figure 2. IgE immunoblot analysis of raw (lane 1) and processed lentil (lanes 2–10) with a serum pool from subjects with specific IgE to lentil: 31.3 kU/L. Arrow indicates the position of the major lentil allergen.

IgE antibody reactivity to 60-min boiled and autoclaved lentil (2.6 atm for 30 min) was further screened using the 25 individual sera (Fig. 3A and B). Boiled lentils had a complex pattern of IgE-binding proteins in the range of 5–107 kDa. The proteins recognized with higher frequency by the sera were: 37 kDa (32%); 50 kDa (32%); and the 45, 48, and 51 kDa bands that are detected by 25% of the sera.

The overall immunoreactivity of lentils was strongly reduced after autoclaving at 2.6 atm for 30 min, although 11 out of 25 tested sera (44%) reacted to immunoreactive proteins, 19 and 23 kDa being the allergens most frequently recognized. Other heat-resistant allergenic proteins (16, 17, 26, 35, 36, 39, 45, and 50 kDa) were still detected by 2 out of 25 sera. Autoclaving lentils at 2.6 atm for 30 min abolished IgE antibody reactivity from these sera to previously detected components such as 48, 37, and 51 kDa.

Table 1 depicts results of skin prick test with raw and thermal-processed extracts (60-min boiled and autoclaved at 2.6 atm for 30 min) of lentils and chickpeas in 12 patients sensitized to legumes. Six patients showed a positive result to raw lentil, but only two patients (no. 5 and 12) reacted to boiled and autoclaved lentil. Patient no. 5 showed higher skin reactivity to boiled lentil in comparison with raw.

3.2 Effects of heating on chickpea allergens

The SDS-PAGE patterns of the protein extracts of raw, boiled, and autoclaved chickpea seeds are shown in Fig. 4. Figure 5 shows the IgE immunoblotting analysis of chickpea extracts using the serum pool. Raw and boiled chickpea up to 30 min have similar SDS (Fig. 4) and IgE-immunoreactive band pattern (Fig. 5). A decrease in the number and intensity of bands is observed after boiling for 60 and 15 min autoclaving (1.2 atm) with several allergenic proteins still

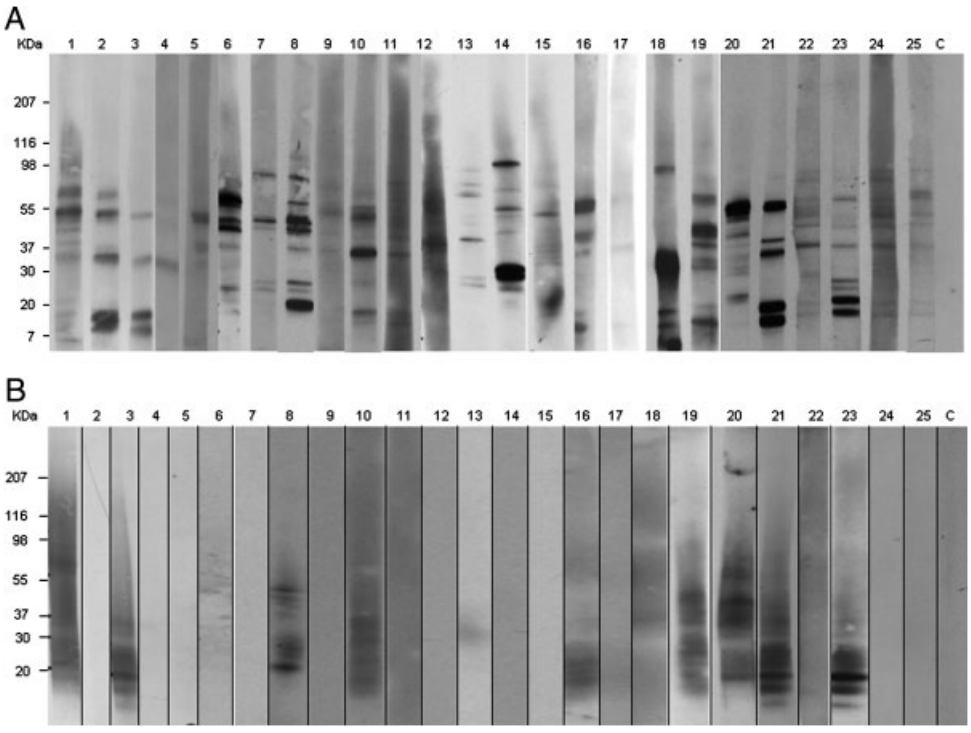

Figure 3. IgE antibody reactivity by immunoblotting to allergens of 60-min boiled (A) and autoclaved lentil (2.6 atm for 30 min) (B). Lentil proteins were resolved by means of SDS-PAGE and transferred to PVDF membranes. All 5-mm strips from each blot were tested for reactivity to serum IgE from 25 subjects sensitized to lentil-specific IgE. Lanes 1–25 represent sera from subjects 1–25, respectively. Lane C represents a negative control serum.

Table 1. Results of skin prick testing with raw and thermal-processed extracts (60-min boiled and autoclaved at 2.6 atm for 30 min) of lentils and chickpeas in patients sensitized to legumes

Patient no.	Specific IgE (kU/L)					SPT (mm) lentil			SPT (mm) chickpea		
	Lentil	Chickpea	Peanut	Soybean	Green bean	Raw	Boiled	Autoclaved	Raw	Boiled	Autoclaved
1	–	0.70	–	–	–	0	0	0	0	0	0
2	–	–	1.70	–	–	0	0	0	0	0	0
3	–	–	0.87	–	–	0	0	0	0	0	0
4	6.13	28.6	4.55	11.8	–	0	0	0	0	0	0
5	0.34	0.48	4.38	2.09	–	3	9.25	3.25	4.75	5.75	6.5
6	0.43	0.12	1.26	–	–	4	0	0	0	0	0
7	0.94	–	1.01	0.62	–	5.75	0	0	0	0	0
8	0	0	–	–	0.35	0	0	0	3.75	0	0
9	1.85	1.30	0.88	0.42	–	6.5	0	0	8	0	0
10	0.62	–	–	–	–	0	0	0	0	0	0
11	0.36	–	1.23	–	–	4.75	0	0	0	0	0
12	7.78	4.60	–	4.22	3.68	6.75	7	6	0	7.25	6.5

visible. Immunoreactivity is strongly reduced after autoclaving and the reduction is higher as pressure and treatment time increase. At 2.6 atm (15 min) four heat-stable-immunoreactive bands are identified (40, 26, 19, and 16 kDa) and after 30 min, two bands (19 and 16 kDa) are still detected.

IgE immunoblots of chickpea, boiled for 60 min and autoclaved at 2.6 atm for 30 min with the individual sera of 24 patients, are shown in Fig. 6A and B. Multiple immunoreactive proteins are identified in boiled chickpeas in the range of 5–124 kDa. The 25 and 42 kDa proteins are recognized by approximately 33% (5 out of 24 sera) of the sera. Other bands are also detected by approximately 21% (8 out of 24 sera) of the sera (21, 23, 27, 31, and 37 kDa).

After autoclaving at 2.6 atm for 30 min, several immunoreactive chickpea proteins are still recognized by 10 out of the 24 sera. The 11 and 28 kDa allergens show the highest recognition frequency (3 sera). The rest of the immunoreactive bands of boiled chickpea extract were eliminated by autoclaving.

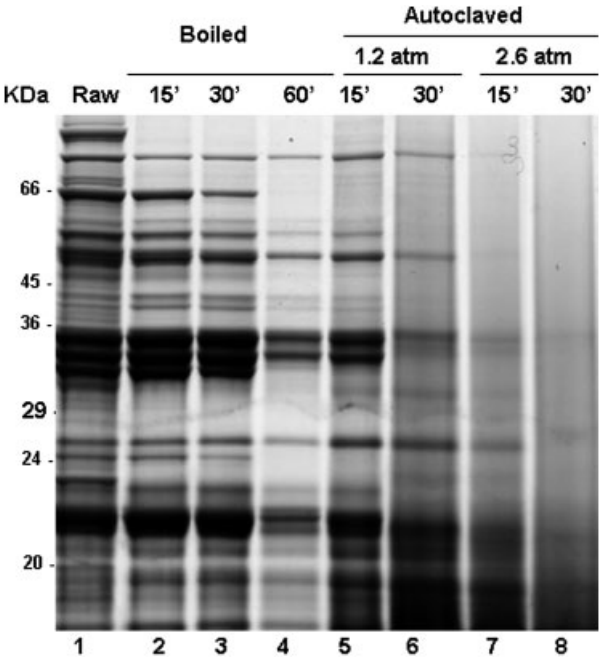

Figure 4. SDS-PAGE patterns of protein extract from raw (lane 1) and processed chickpea (lanes 2–8) samples.

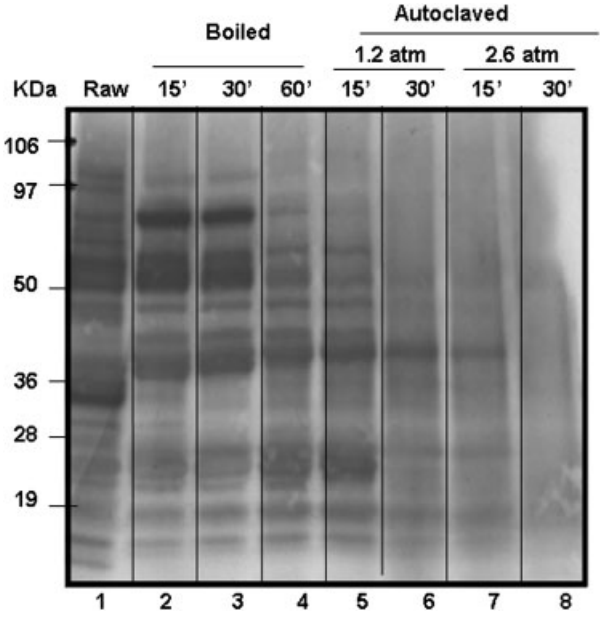

Figure 5. IgE immunoblot analysis of raw (lane 1) and processed chickpea (lanes 2–8) with a serum pool from subjects with specific IgE to chickpea: 29.0 kU/L.

Skin testing with raw extract elicited a positive response in 3 out of 12 patients, whereas boiled and autoclaved extracts elicited positive reactions in two patients (Table 1). Patient no. 12 showed a negative skin prick result to raw chickpea, while reacting to boiled and autoclaved extracts.

4 Discussion

Thermal and nonthermal processing can increase or decrease allergenicity of food proteins or result in new allergens (neoallergens) being formed. Therefore, the study of food processing seems to be important to evaluate food allergenicity [28]. Most investigations dealing with the influence of thermal processing on legume allergenicity have been focused on peanut and soybean [14, 29–32]. Several authors have described an increase in peanut allergenicity with roasting procedures [14, 15, 33, 34]. Beyer *et al.* [14] observed that in contrast to roasting, frying and boiling peanuts reduced IgE-binding reactivity to the major peanut allergens Ara h 1, Ara h 2, and Ara h 3. Burks *et al.* [29] did not find a significant decrease of IgE binding after heating (100°C, up to 60 min) soybean protein extracts. However, boiling for 120 min and microwave heating of soybean seems to decrease allergenicity, as only half of soybean-allergic patients were shown to have detectable specific IgE against heated soybean protein [35].

The present results demonstrated that boiling treatments produce little modifications on SDS-PAGE pattern (Figs. 1 and 4) and IgE-binding capacity (Figs. 2 and 5) of lentil and chickpea allergenic proteins. In fact, the presence of multiple IgE-binding proteins when 60-min boiled lentil and chickpea extracts were tested with individual sera (Figs. 3A and 6A) demonstrate that both lentil and chickpea still contain a relevant number of heat-stable-immunoreactive proteins.

To date, there are few studies describing the effect of heat treatment on lentil and chickpea allergenicity. Boiling of lentil seeds did not reduce immunoreactivity as demonstrated by ELISA inhibition experiments and immunoblotting [11, 19]. Martinez *et al.* [10] analyzed the allergenic composition of raw and boiled chickpea extract detecting similar IgE-binding proteins in both. Our results confirmed the thermo stability of lentil and chickpea allergens.

The present findings suggest that autoclaving produces a severe effect on the integrity and structure of lentil and chickpea proteins, which runs in parallel with a decrease in IgE-binding properties of these legume seeds. To date, little is known about the effect of the pressure technique process, *i.e.* autoclaving on allergenic proteins. Venkatachalam *et al.* [16] and Brenna *et al.* [17] demonstrated that the allergenicity of almond proteins and peach nectar (Pru p 1) protein is maintained after autoclaving at 1.2 atm (121°C) during 30 min while allergenicity of green pea is reduced after autoclaving at 121°C for 15 min [18]. In our previous studies lupine allergenicity could be abolished by autoclaving at 2.6 atm for 30 min or by DIC at 6 bar (5.9 atm) during 3 min, whereas it was only slightly affected by boiling, microwave, and extrusion-cooking, demonstrating the high thermal resistance of the major lupine allergens [20, 21].

From our point of view, aggregation and differential solubility cannot totally explain the large amount of modifications of allergenic proteins observed upon processing, especially autoclaving, in contrast to what reported by Mondoulet *et al.* [15]. Our data are more in agreement with an extensive

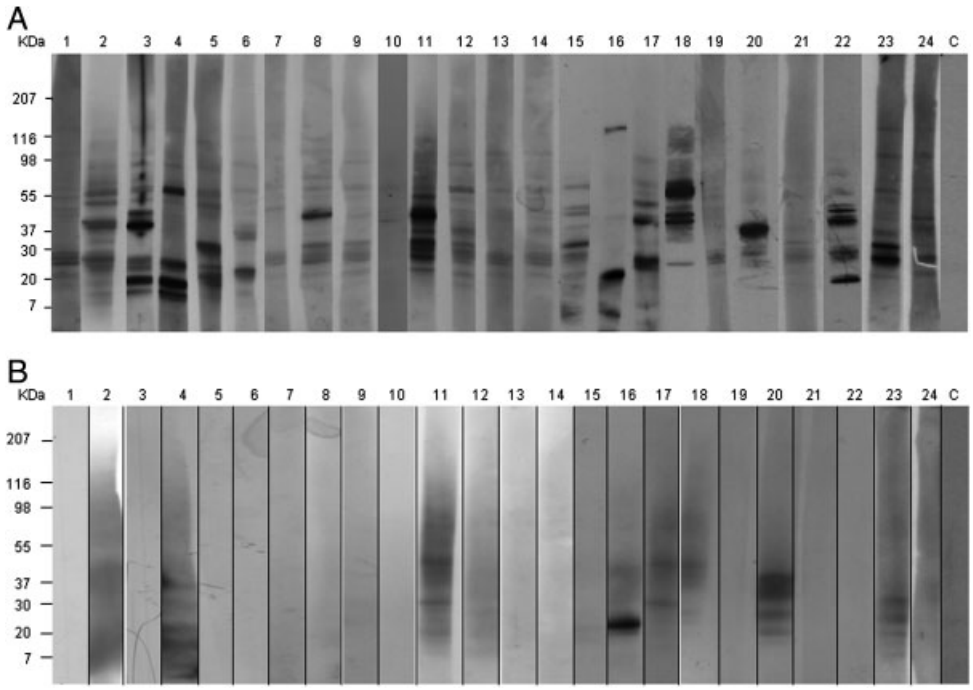

Figure 6. IgE antibody reactivity by immunoblotting to allergens of 60-min boiled (A) and autoclaved chickpea (2.6 atm for 30 min) (B). Chickpea proteins were resolved by means of SDS-PAGE and transferred to PVDF membranes. All 5-mm strips from each blot were tested for reactivity to serum IgE from 24 subjects sensitized to chickpea specific IgE. Lanes 1–24 represent sera from subjects 1–24, respectively. Lane C represents a negative control serum.

degradation of the proteins that would produce short peptides situated in the low MW portion of the gels, causing the smear observed in the low-MW portions of electrophoretic lanes of autoclaved samples (Figs. 1, 2, 4, and 6). This could explain the disappearance of many IgE-immunoreactive band proteins [28]. We observed that the IgE immunoreactivity of lentil and chickpea extracts only decreased significantly at extreme conditions, when the maximum pressure and time treatment (autoclaving at 2.6 atm during 30 min) were applied. When autoclaved, lentil samples at harsh conditions were analyzed by immunoblot with individual sera or a serum pool, the putative lentil major allergen Len c 1 could not be detected, although some extremely resistant bands still conserved the IgE-binding capacity (Figs. 2 and 3). The number of sera reacting to IgE-reactive proteins in autoclaved lentil extract is higher in comparison with autoclaved chickpea, a fact that suggests that the thermal stability of lentil allergens is stronger than that of chickpea allergens.

In summary, we can conclude that autoclaving at 2.56 atm for 30 min produces a significant decrease of IgE-binding capacity of lentil and chickpea allergens, as less than half of individual sera and patients react against these extremely treated legume seeds. However, several immunoreactive proteins still remained in these legumes upon harsh autoclaving. Further work on their identification would need to be undertaken.

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The authors have declared no conflict of interest.

5 References

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