

Boiling and Pressure Cooking Impact on IgE Reactivity of Soybean Allergens

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Keywords

Boiling · Legume allergy · Pressure cooking · Skin-prick testing · Soybean allergy · Thermal processing

Abstract

Background: Soybean is one of the 8 foods that causes the most significant rate of food allergies in the USA and Europe. Thermal processing may impact on the allergenic potential of certain foods. We aimed to investigate modifications of the IgE-binding properties of soybean proteins due to processing methods that have been previously found to impact on the allergenicity of legumes such as peanut. **Methods:** Soybean seeds were subjected to different thermal processing treatments. To evaluate their impact on the IgE-binding capacity of soybean proteins, individual sera from 25 patients sensitized to soybean were used in in vitro immunoassays. Detection of specific soybean allergens in untreated and treated samples was carried out with specific monoclonal and polyclonal antibodies. In vivo studies of skin prick testing (SPT) were also performed. **Results:** The IgE reactivity of soybean was resistant to boiling up to 30 min, and this treatment had a higher impact when applied for 60 min. Treatment that combined heat and pressure produced a fragmentation of proteins in both soluble and insoluble frac-

tions that went along with a decreased capacity to bind IgE and reduced the SPT wheal size. However, allergens such as 7S globulins survived this treatment. **Conclusions:** Thermal-processing methods able to attenuate the capacity of soybean proteins to bind IgE may contribute to the improvement of food safety and could constitute a potential strategy for the induction of tolerance to soybean.

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Introduction

Soybean is a member of the Leguminosae family, and one of 8 foods responsible for the most significant allergic reactions to foods in the USA and Europe. Allergic reactions to soybean were associated in high proportion to peanut allergy in an international multicenter study conducted in Europe [1, 2]. Soybean proteins are present in a wide variety of food products, and in undeclared traces, as hidden allergens. This can be a threat for patients with soybean allergy [3]. Soybean consumption has increased over the years due to its nutritional and health benefits

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[4–6]. Soybean products consumed nowadays are numerous, and include tofu, miso, tempeh, soybean sauce, texturized soybean protein, etc. The process involved in the production of the different soybean products includes cooking, grinding, fermenting, sprouting, etc. Soybean proteins are an important source of essential amino acids and get added to a wide variety of processed foods such as meat, bakery products, cereals, and chocolate [2, 4].

The processing methods to which soybean is subjected have the potential to alter the biochemical characteristics of the proteins, or induce chemical reactions within the components of the food matrix, with a possible impact on soybean allergenicity [7–9]. Franck et al. [10] found that texturized soybean protein, the production of which involves heating, mechanical pressure, and acid treatment, presented a distinctive IgE-binding protein profile (when compared with soybean flour or soybean milk), with decreased IgE recognition of proteins of a molecular weight of 30–34 kDa. A total decrease of IgE-binding proteins in a soybean milk infant formula was observed as well.

Thermal treatments, such as boiling, alter the allergenicity of legumes such as peanut, due to the transfer of low-molecular-weight peanut allergens, such as Ara h 2, Ara h 6, or Ara h 7, from the seeds to the boiling water [11, 12]. It has been found that boiling for 12 h in a closed vessel led to a 19-fold decrease of the IgE-binding capacity of peanut proteins [13]. Pressure cooking at specific conditions has also been found to impact on the IgE-binding properties of legumes [9, 14–16]. However, the effects of boiling or pressure cooking on soybean IgE reactivity have not yet been thoroughly explored and analyzed. The use of soybean with a decreased capacity to bind IgE would be of considerable importance for the industry and consumers, and might represent a potential strategy for immunotherapy in patients with severe anaphylaxis to soybean. The aim of this study was to investigate modifications in the IgE-binding properties of soybean proteins due to processing methods previously found to impact on the allergenicity of legumes such as peanut.

Material and Methods

Patients and Sera

A pool of sera with specific IgE to soybean (13.9 kU/L) was used for the Western blot IgE experiments. Additionally, individual sera from 25 patients with IgE reactivity to soybean were utilized. After providing their informed consent, 5 additional patients with specific serum IgE to soybean, ranging from 0.42 to 11.8 kU/L, underwent skin prick testing (SPT) with untreated and treated soybean

according to standard methods [17]. As a control, 2 healthy subjects were tested. The study was approved by the Ethics Committee of the Hospital Universitario 12 de Octubre, Madrid, Spain (permission No. 0312150129).

Plant Material, Treatments, and Conditions Applied

Soybean seeds (*Glycine max*, variety Aires) were obtained from the Italian Society of Seeds (Bologna, Italy). The first treatment was boiling at different time points. For this purpose, soybean seeds (1:5 w/v) were placed in boiling distilled water (100°C) for 15, 30, and 60 min. For the pressure cooking treatment, a tabletop autoclave (CertoClav Multicontrol IPX4, Traun, Austria) was used. Soybean seeds (1:5 w/v of distilled water) were treated at 121°C and 1.18 atm air gauge pressure, for 15 (i.e., autoclaving 1 [AU1] treatment) or 30 min (AU2 treatment), and at 138°C and 2.56 atm, for 15 (AU3 treatment) or 30 min (AU4 treatment) (online suppl. Fig. 1; see www.karger.com/doi/10.1159/000485894 for all online suppl. material).

Untreated and treated seeds were then milled to pass through a 1-mm sieve (Tecator, Cylotec 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. Protein extracts were obtained from defatted flours 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 extracts were clarified by centrifugation at 27,000 g for 30 min at 4°C, and the supernatants were dialyzed against distilled water during 24 h at 4°C using a dialysis membrane (Spectra/Por, Serva, Heidelberg, Germany) with a cut-off of 3.5 kDa, and then freeze-dried. The bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL, USA) was used for protein quantitation.

Solubilization Analysis by Protein Electrophoresis

To compare the electrophoretic pattern of soluble protein extracts obtained from untreated and treated soybean flour with total protein obtained by means of direct solubilization of the flours in an electrophoresis SDS sample buffer, samples were electrophoresed and subjected to Coomassie blue staining for analysis, as previously described [14].

IgE Western Blot with Serum Pool and Individual Sera

Protein extracts from untreated and treated soybean (20 µg protein/lane) were electrophoresed and transferred to PVDF membranes as previously described [15]. After blocking with 5% BSA in PBS, membranes were subjected to overnight incubation with a serum pool or individual sera (1:10) at 4°C. For incubation with individual sera, membranes were cut into 3-mm strips, in order to be incubated individually with each serum. Afterwards, they were washed and treated with mouse anti-human IgE monoclonal antibody (clone HE2/1) (1:3,000) for 2 h [18]. After washing, they were incubated with a rabbit anti-mouse IgG horseradish peroxidase (HRP)-conjugated antibody (1:5,000) (Dako, Glostrup, Denmark) for 1 h. Detection was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (GE Healthcare Life Sciences, Little Chalfont, UK).

Detection with Monoclonal Antibodies by Western Blot, ELISA, and Dot Blot

IgG mouse anti-Ara h 1 (clone 2C12, stock 2.7 mg/mL), anti-Ara h 2 (clone 1C4, stock 2 mg/mL), anti-Ara h 3 (clone 1E8, stock

Table 1. SPT results from testing 5 patients (A–E) sensitized to soybean (specific serum IgE to soybean ranging from 0.42 to 11.8 kU/L) and 2 healthy controls

Patient	Specific IgE to soybean, kU/L	Soybean SPT, mm			Positive control ^a SPT, mm	Negative control ^b SPT, mm
		untreated	after boiling for 60 min	after AU4 treatment		
A ^c	11.8	4	0	0	6.2	0
B ^d	2.09	3.25	0	0	6	0
C ^d	0.42	0	2	0	6	0
D ^d	4.22	4	4	3.75	6.5	0
E ^d	0.45	3	0	0	4.5	0
Control 1	0	0	0	0	4.5	0
Control 2	0	0	0	0	5	0

The SPT result was the measurement of the diameter of the wheal formed.

^a Histamine dihydrochloride. ^b Saline solution. ^c Positive double-blind placebo-controlled food challenge (DBPCFC) with soybean. ^d Negative DBPCFC or open food challenge with soybean.

1.1 mg/mL), and anti-Ara h 8 (clone 4G6, stock 1 mg/mL) monoclonal antibodies (Indoor Biotechnologies, Charlottesville, VA, USA) were tested with protein extracts from untreated and treated soybean by Western blot, ELISA, and Dot blot.

For Western blot, proteins (20 µg protein/lane) were electrophoresed and transferred to PVDF membranes. Blocking was performed for 1 h in Tris-buffered saline (TBS) containing 0.5% Tween-20 (TBST) and 5% nonfat milk (blocking solution). Anti-Ara h 1, anti-Ara h 2, anti-Ara h 3, or anti-Ara h 8 antibodies were diluted in blocking solution (1:5,000) and incubated overnight at 4°C with the PVDF membranes. After a washing step, the membranes were treated with HRP-conjugated goat anti mouse antibody (stock: 0.4 mg/mL, diluted at 1:5,000) (Santa Cruz Biotechnology, Dallas, TX, USA). Detection was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (GE Healthcare). The signal was measured using ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare).

ELISAs were performed in polystyrene high-bind microplates (Corning Inc., NY, USA). Wells were coated with 100-µL/well of protein extracts from untreated or treated soybean (50 µg/mL in PBS), and incubated overnight. After a washing step, they were blocked with PBS containing 3% nonfat milk plus 0.1% Tween-20. Plates were incubated with anti-Ara h 1, anti-Ara h 2, anti-Ara h 3, or anti-Ara h 8 antibodies (1:2,500) for 2 h, washed, and then incubated with HRP-conjugated goat anti mouse antibody for 1 h. After washing, the peroxidase reaction was developed with a tetramethylbenzidine (TMB) substrate (substrate reagent pack, DY999, R&D Systems, Inc., Minneapolis, MN, USA). 2 N H₂SO₄ was used to stop the reaction, and the OD was measured at 450 nm. Wells, coated with blocking solution instead of soybean protein samples and incubated directly with the primary and secondary antibodies, were used as negative controls. All the tests were performed in duplicate. The formula: mean (OD) + 3 × SD was calculated for the negative controls in order to establish the cut-off point of positivity.

Dot blot was performed by applying samples (10 µg protein per dot) on PVDF membranes, blocking in blocking solution, and in-

cubating overnight with anti-Ara h 1, anti-Ara h 2, anti-Ara h 3, or anti-Ara h 8 antibodies (1:2,500). The rest of the assay was performed as described above for Western blot.

Detection with Polyclonal Antibodies by Western Blot and ELISA

IgG rabbit anti-Ara h 1 polyclonal antibody (stock 3 mg/mL, MyBioSource, San Diego, CA, USA) and IgG rabbit anti-Ara h 2 polyclonal antibody (stock 2 mg/mL, Indoor Biotechnologies) were additionally tested with protein extracts from untreated and treated soybean by Western blot and ELISA. Similar procedures to the experiments with monoclonal antibodies described above were used, with the exception of HRP-conjugated donkey anti-rabbit secondary antibody, were used here (Santa Cruz Biotechnology).

Skin Prick Testing

SPT was carried out after performing the in vitro experiments. Five subjects sensitized to soybean (specific serum IgE to soybean ranging from 0.42 to 11.8 kU/L; Table 1, individual data) were asked to undergo SPT with untreated and treated soybean extracts (10 mg/mL protein in PBS). SPT was performed in duplicate on the volar side of the forearm according to standard methods [17]. The wheal diameters were measured after 15 min. A mean diameter of ≥3 mm compared with that of the negative control was considered as positive result. The positive control was histamine dihydrochloride (10 mg/mL) and the negative control was saline solution.

Results

Solubilization Experiments

In order to analyze a possible loss of protein solubility during the treatments applied in this study, we extracted total protein by direct solubilization of the defatted soy-

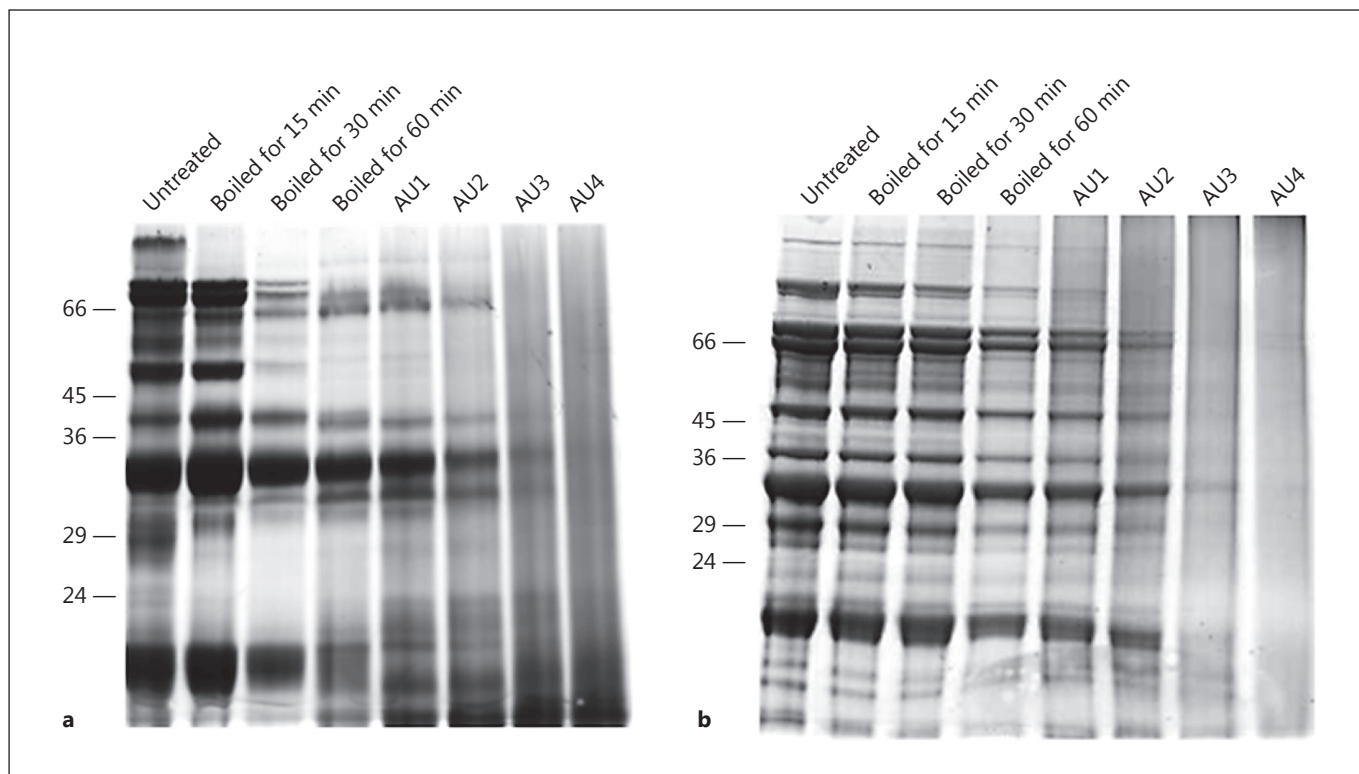


Fig. 1. Electrophoretic comparison (SDS-PAGE) of soluble protein extracts obtained from untreated and treated soybean (boiled and AU-treated) (**a**), and of total protein obtained by direct solubilization of soybean flours in SDS electrophoresis sample buffer (**b**).

bean flours in SDS electrophoresis sample buffer, and compared the protein profiles of both soluble protein obtained by extraction with 0.1 M PBS buffer (pH 7.4) plus 0.15 M NaCl and total protein obtained by direct solubilization. Results showed similar effects of the treatments on the protein profiles of both fractions. AU treatments (especially AU3 and AU4) led to a similar protein profile of both fractions (Fig. 1).

IgE-Binding Patterns of Soybean Subjected to Boiling and Pressure Cooking

The effects of boiling and pressure cooking (AU treatments) on the IgE-binding properties of soybean proteins were evaluated. A serum pool from patients with specific IgE to soybean (13.9 kU/L) was used to identify changes in the IgE-binding protein profiles by Western blot. Multiple bands were detected in the untreated soybean sample by the serum pool (Fig. 2a). Boiling for 15 and 30 min did not significantly change the IgE-binding protein profile. However, soybean boiled for 60 min contained less IgE-binding proteins, although several heat-stable pro-

teins were still detectable by means of the IgE of the serum pool. AU treatments progressively decreased IgE-reactive proteins of soybean as increased pressure, temperature, and time were applied. An increase of protein degradation with a decrease of IgE-binding properties was observed by Western blot of these samples, especially in the case of AU3 and AU4 treatment.

Sera from 25 patients with IgE reactivity to soybean were used to identify the individual profiles of allergens in untreated and AU4-treated soybean (Fig. 2b, c). Multiple immunoreactive proteins (molecular-weight range, 12–120 kDa) were identified in untreated soybean. After the AU4 treatment, a strong change of the allergen profile could be found, but some immunoreactive proteins were still recognized by several sera.

Detection of Soybean Allergens with Specific Monoclonal and Polyclonal Antibodies in Untreated and Treated Samples

Specific antibodies were used to analyze changes of soybean allergens in untreated and treated samples. Due

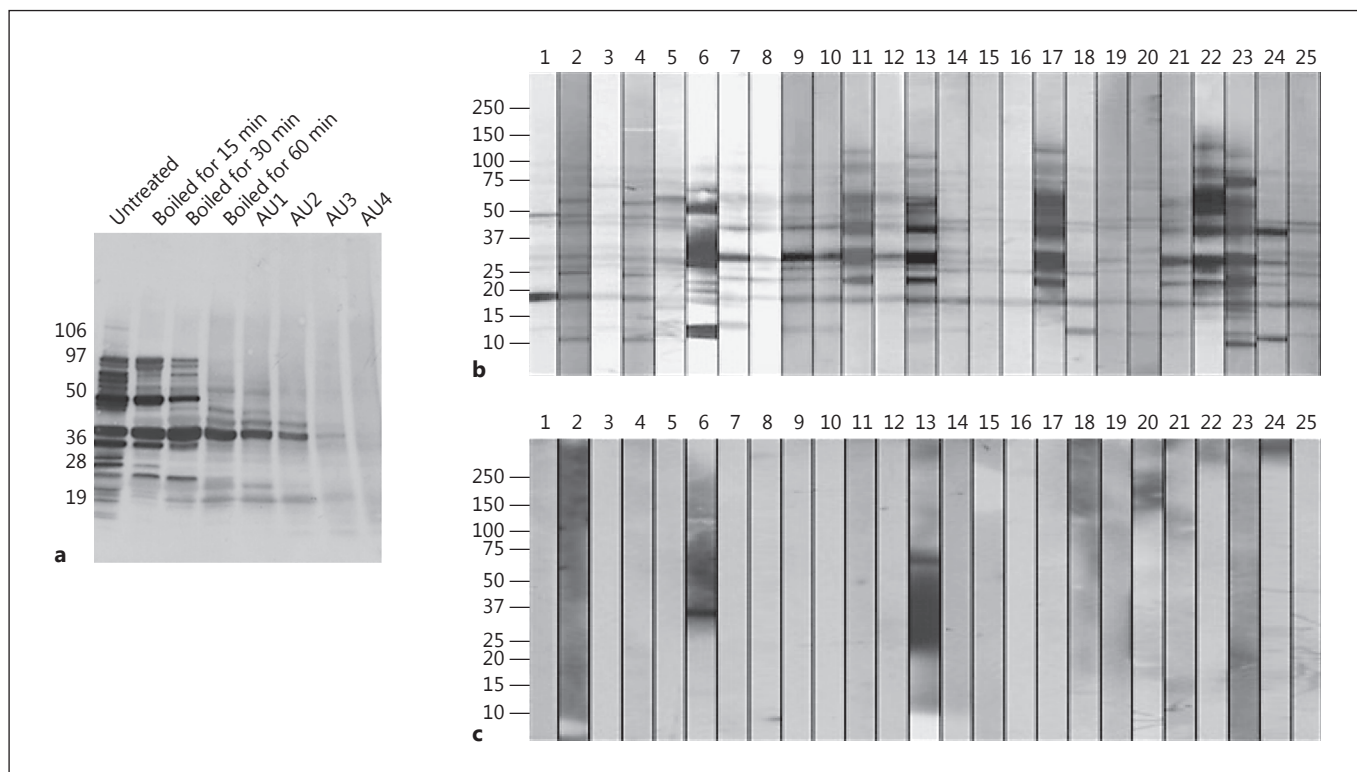


Fig. 2. **a** Analysis of untreated or treated (boiled and AU-treated) soybean by IgE-Western blot. The membrane was incubated with a serum pool with specific IgE to soybean (13.9 kU/L), and then processed as indicated in Materials and Methods. **b, c.** Recognition of IgE-binding proteins of untreated soybean (**b**) and soybean subjected to AU4 treatment (**c**) by Western blot using individual sera. Membranes were incubated with 25 individual sera with IgE reactivity to soybean, and then processed as indicated in Material and Methods.

to similarities between certain soybean and peanut allergens, and the scarcity of available antibodies against soybean allergens, we utilized monoclonal anti-Ara h 1, anti-Ara h 2, anti-Ara h 3, and anti-Ara h 8 antibodies to analyze changes in soybean allergen detection due to the thermal treatments used in this study. The results showed that the antibodies strongly recognized untreated soybean allergens by Western blot, ELISA, and Dot blot (Fig. 3a–e). Monoclonal anti-Ara h 1, 2, 3, and 8 recognize 7S globulin, 2S albumin, 11S globulin, and Bet v 1-like protein in soybean, respectively. In the case of the boiled soybean samples, the 4 antibodies showed a decreased detection of soybean allergens with an increase in the time of boiling, up to 60 min. AU treatments applied to soybean produced a marked decrease of detection with monoclonal anti-Ara h 2, anti-Ara h 3, and anti-Ara h 8 antibodies. However, a decreased but still positive detection with monoclonal anti-Ara h 1 antibody of AU-treated soybean (especially AU3 and AU4) in ELISA and Dot blot could be found (Fig. 3a, e).

We additionally used polyclonal antibodies (anti-Ara h 1 and anti-Ara h 2) to further analyze changes in the detectability of soybean allergens from the treatments considered in this study. As expected, wider recognition was achieved with polyclonal antibodies. However, a similar decrease in the detectability of allergens from boiled and AU-treated soybean could be achieved with polyclonal anti-Ara h 2 (online suppl. Fig. 2). Interestingly, polyclonal anti-Ara h 1 antibody, which recognizes 7S globulin in soybean, but also recognizes a breakdown product of this allergen, showed a decreased detectability of soybean allergens in the sample boiled for 60 min. Similar to the results with monoclonal anti-Ara h 1, the polyclonal antibody showed a decreased but still positive detectability of AU3- and AU4-treated soybean (online suppl. Fig. 2).

Skin Prick Testing

We were also interested in analyzing the effects of the treatments on the reactivity of soybean allergens by means of SPT. We selected 3 soybean samples to perform SPT:

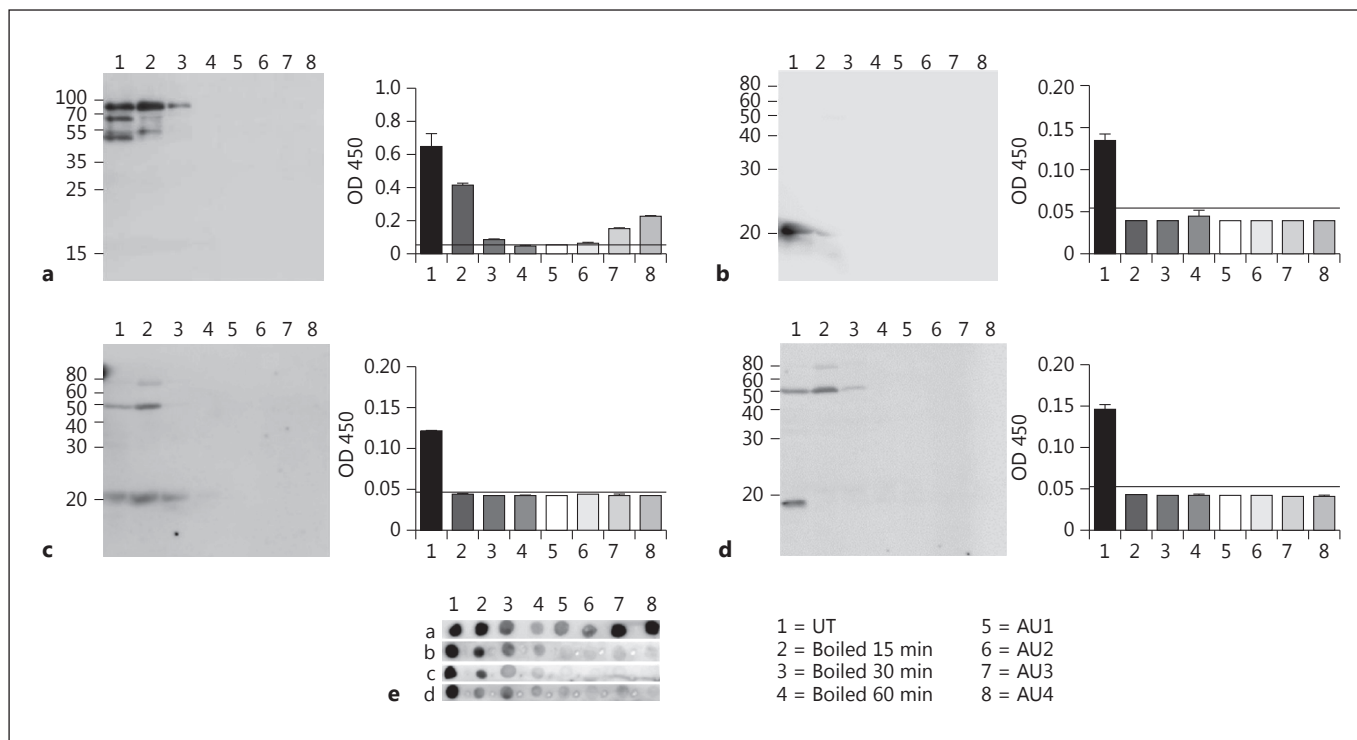


Fig. 3. Detection of soybean allergens in untreated (UT) and treated soybean with monoclonal anti-Ara h 1 (a), anti-Ara h 2 (b), anti-Ara h 3 (c), and anti-Ara h 8 (d) antibodies by Western blot and ELISA. e A Dot blot with untreated and treated soybean incubated with anti-Ara h 1 (a), anti-Ara h 2 (b), anti-Ara h 3 (c), and anti Ara h 8 (d) antibodies was also performed.

untreated, boiled for 60 min, and AU4-treated (2.56 atm, 138°C, 30 min), in addition to positive (histamine dihydrochloride) and negative (saline solution) controls. Table 1 shows that soybean boiled for 60 min decreased the wheal size compared to untreated soybean in 3 out of 5 patients (“A,” “B,” and “E”). In 2 out of 5 patients, soybean boiled for 60 min did not change the wheal size compared with untreated soybean (patient “D”), or it increased the wheal size from 0 to 2 mm (patient “C”). AU4 treatment was able to decrease the wheal size to 0 mm in 4 out of 5 patients (“A,” “B,” “C,” and “E”). The fifth patient (“D”) showed a slight decrease from 4 mm with untreated soybean to 3.75 mm with AU4-treated soybean.

Discussion

Nowadays, soybean allergy is an important health problem. Despite the numerous processing methods that soybean is subjected to, only a few studies have evaluated the effects of different processing methods on its allergenicity [16, 19, 20].

For this study, we selected boiling and pressure cooking (AU treatment) under different conditions to evaluate their impact on the IgE reactivity of soybean. The results showed that the IgE reactivity of soybean resisted boiling for 15 and 30 min, as assessed by Western blot using sera from patients sensitized to soybean. Boiling for 60 min decreased the IgE reactivity of soybean. However, AU treatments were more efficient in decreasing the IgE reactivity of soybean as the conditions of temperature, pressure, and time were increased. The strongest effect was achieved at the highest pressure (2.56 atm), temperature (138°C), and time (30 min) applied (AU4 treatment). Soybean subjected to such treatment used for SPT led to a reduced wheal size in most of the patients, indicating that the decrease of IgE reactivity seen on Western blot went along with a decreased capacity to cross-link IgE and to produce a positive result on SPT. Several studies have previously found that the heat and pressure involved in pressure cooking under specific conditions can produce a fragmentation of the proteins from certain foods, with the potential to decrease their IgE-

binding properties [14, 15, 21, 22]. Interestingly, it seems that pressure alone or high temperature alone do not affect the IgE-binding properties of foods in the same way as the combination of both of these factors. For instance, walnut was found to be resistant to conditions of high pressure at low temperatures. However, a combination of pressure (2.56 atm) and a temperature of 138°C led to a fragmentation of walnut proteins with a decreased capacity to bind and cross-link IgE [23].

Only a few studies have evaluated the effects of thermal processing on the IgE reactivity of soybean [16, 19, 20]. However, several studies have evaluated the effects of different thermal-processing methods on the allergenicity of other legumes (e.g., lupin or peanut) or tree nuts. Alvarez-Alvarez et al. [21] and Guillamón et al. [24] found that lupin subjected to autoclaving (2.56 atm, at 138°C for 30 min) or instantaneous controlled pressure-drop treatment (5.9 atm, for 3 min) showed a decrease in IgE reactivity whereas treatments such as boiling, extrusion, or microwave heating had minor effects on its IgE reactivity. Other authors have found that among treatments such as microwave heating, boiling, or roasting, pressure cooking at 121°C and 15 psi for 20 min was the only treatment that decreased the detectability of the cashew allergens Ana o 1, Ana o 2, and Ana o 3 [25]. Similar effects were found for pecan [26].

We analyzed changes in the detectability of specific soybean allergens due to boiling and AU treatments using specific monoclonal and polyclonal antibodies. We found a decrease in the recognition of 2S albumin (putative Gly m 8) in boiled and AU-treated soybean using both monoclonal and polyclonal antibodies. The detection of 11S globulin (putative Gly m 6) and Bet v 1-like protein (putative Gly m 4) in boiled and AU-treated soybean samples using a monoclonal antibody was also decreased. The detection of 7S globulin (putative Gly m 5) with a monoclonal antibody, however, was still positive for soybean samples subjected to the strongest conditions of heat and pressure (AU3 and AU4 treatments). This result was confirmed using a polyclonal antibody. Previous studies have found that during extensive boiling in a closed vessel, certain allergens (especially 2S albumins) can be transferred from the seed to the cooking water in peanut, decreasing, in this way, their content in the seed [12, 13]. Other allergens such as 7S globulins, however, are more resistant to thermal processing, and can form large complexes during heating [27]. These factors may explain, in part, the decrease of certain allergens, such as 2S albumins or 11S globulins, in soybean, after boiling for 60 min or after AU treatment. However, a higher resistance of other soybean

allergens such as 7S globulins in AU3- and AU4-treated-soybean was found.

In this study, we examined protein extracts obtained from untreated and treated soybean flours and found a marked decreased IgE reactivity in soybean treated under strong conditions of heat and pressure (AU3 and AU4 treatments). It may be hypothesized that the protein extracts obtained from thermal treated-samples contain less IgE-binding proteins by a decrease of protein solubilization due to increased conditions of heat and pressure. However, we analyzed untreated and treated soybean flours directly solubilized in SDS electrophoresis sample buffer, in which almost total protein solubilization can be obtained. We found similar patterns of protein fragmentation due to AU treatments in both soluble protein extracts and total protein. Therefore, the alterations induced by thermal treatments in the soluble soybean protein extracts are representative for the global alterations of such thermal treatments on soybean total protein.

The potential use of soybean with a decreased capacity to bind IgE would be of considerable importance for the industry and consumers, but also constitute a potential strategy for oral immunotherapy in patients who experience severe anaphylaxis to soybean. Our research forms the basis for further studies on the potential use of heat/pressure-treated soybean with a decreased IgE reactivity in immunotherapy.

In conclusion, we found that IgE reactivity of soybean is not affected by boiling for up to 30 min, but is partially decreased by boiling for 60 min. Heat and pressure treatments led to the highest decrease of soybean IgE reactivity, due to a potential protein fragmentation, although some soybean allergens (such as 7S globulins) remained after treatment. Soybean with a decreased capacity to bind IgE may contribute to the improvement of food safety and could constitute a potential strategy for tolerance induction to soybean.

Acknowledgements

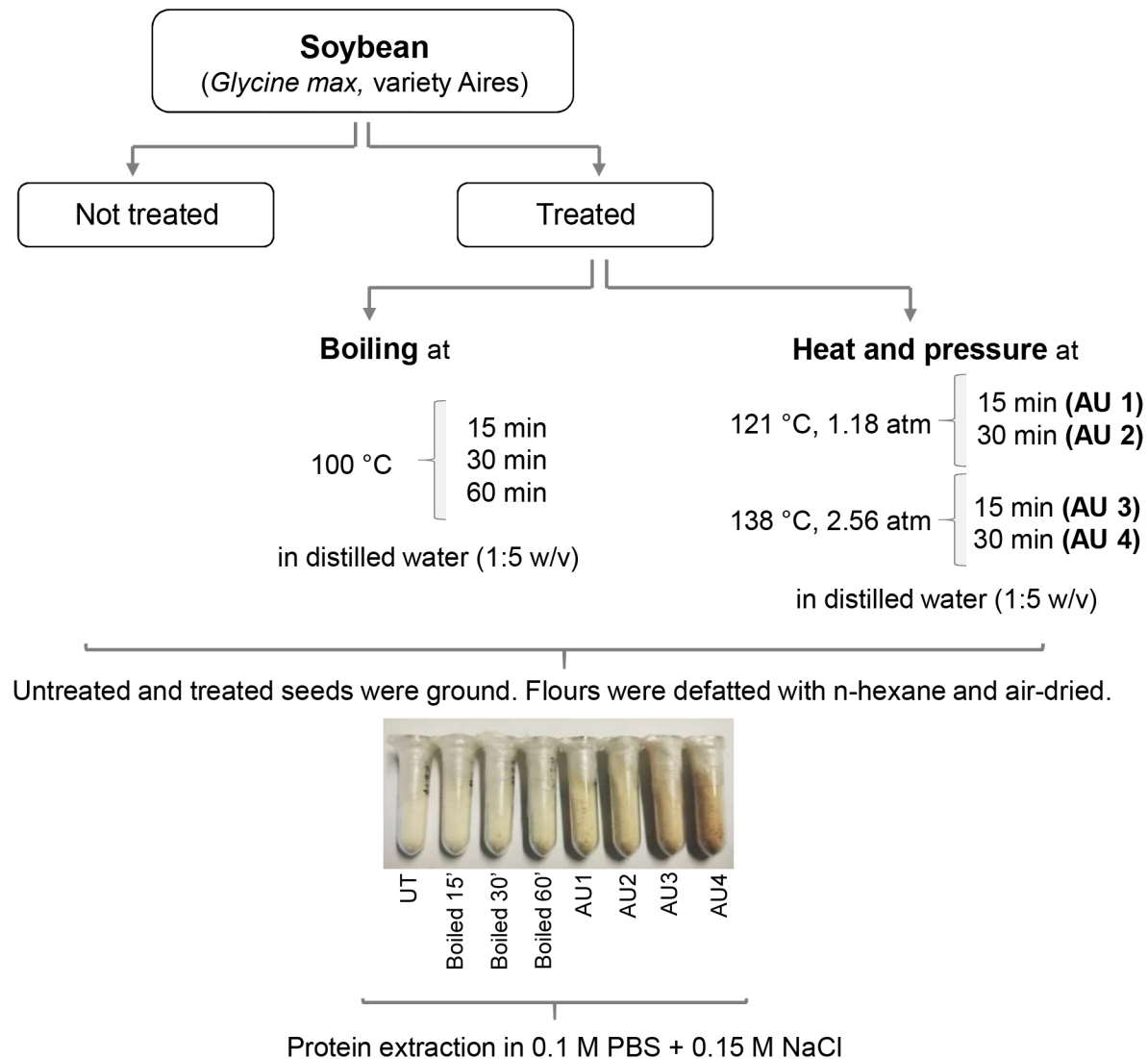
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Disclosure Statement

The authors declare no conflicts of interest.

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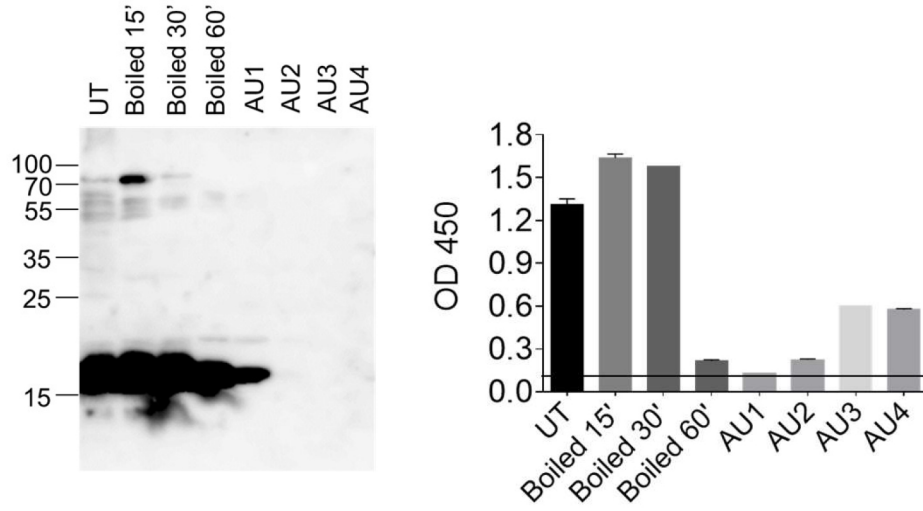
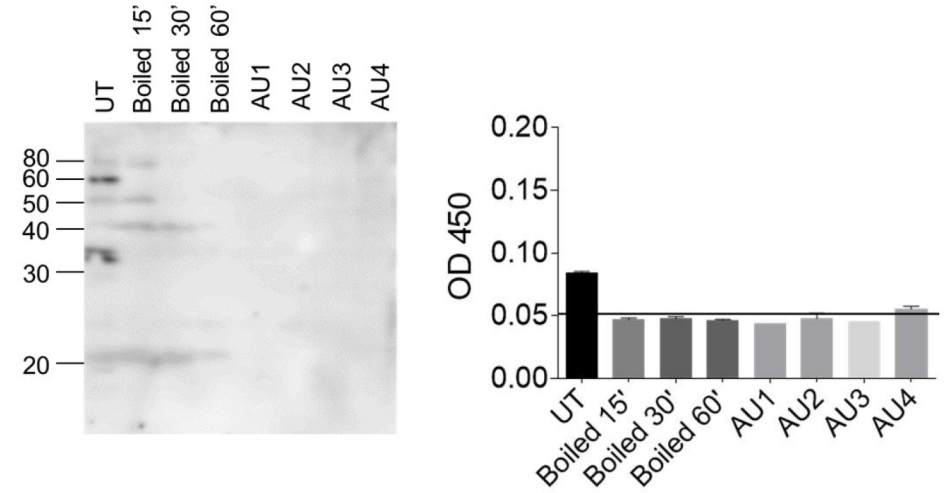
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Supplementary material. Figure 1. Schematic representation of the plant material used in the study, treatment types, and conditions applied for sample processing.

Suppl. Fig. 1. Schematic representation of the plant material used in the study, treatment types, and conditions applied for sample processing.

Suppl. Fig. 2. Detectability of soybean allergens with polyclonal anti-Ara h 1 (**A**) and polyclonal anti-Ara h 2 (**B**) antibodies in untreated and treated soybean in western blot and ELISA.

A**B**

Supplementary material. Figure 2. Detection of soybean allergens with polyclonal anti-Ara h 1 (A) and anti Ara h 2 (B) antibodies in untreated and treated soybean in western blot and ELISA.