

Effect of an Instantaneous Controlled Pressure Drop on in vitro Allergenicity to Lupins (*Lupinus albus* var Multolupa)

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Key Words

DIC® • Food allergy • In vitro IgE binding • Instantaneous controlled pressure drop • *Lupinus albus* • Thermal processing

Abstract

Background: Lupin seed flour has been reported as a causative agent of allergic reactions, especially in patients with allergy to peanut. Previous studies have demonstrated that autoclave treatment can considerably reduce the allergenicity of lupins. **Aims:** The aim of this work was to evaluate the effect of instantaneous controlled pressure drop (détente instantanée contrôlée, DIC) treatment on lupin in vitro allergenicity. **Methods:** Lupin cotyledons were subjected to instantaneous controlled pressure drop at several pressure and time conditions (3, 4.5 and 6 bar for 1, 2 and 3 min, respectively). Immunoreactivity to raw and DIC-treated extracts was evaluated by Western blot using a serum pool from 19 sensitized patients. **Results:** Depending on the operating parameters used during DIC treatment, a reduction in protein solubility of lupin seed was observed. Moreover, drastic modifications in protein profiles were observed after

DIC treatment by SDS-PAGE analysis. Western blot experiments showed that the decreases in IgE binding to lupin proteins were associated with the increases in steam pressure and time treatment, and binding was completely abolished by DIC at 6 bar for 3 min. **Conclusions:** The results suggest that DIC treatment could produce a reduction in lupin allergenicity.

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Introduction

Plant proteins in general and legumes in particular are being increasingly considered as food ingredients in the last years because of their nutraceutical properties mainly related to the prevention of cardiovascular disease, diabetes and obesity [1]. Sweet white lupins are legumes whose seeds contain a protein content similar to soybean [2]. In addition, lupin is not only interesting in human nutrition for its nutritional value but also for functional properties in bakery, confectionery and meat products [3–5].

The major storage proteins of lupin seeds are globulins (87%), which contain two major protein types, conglutin β (vicilin-like protein, 43.4%), conglutin α (legumin-like protein, 33%) and two minor components, conglutin δ (12.5%) and conglutin γ (6%) [6]. Recent studies have shown that lupin globulin proteins are a hypocholesterolemic agent and potentially reduce the risk of cardiovascular disease [7], and the conglutin γ fraction reduces glucose levels [8].

On the other hand, lupin protein is able to induce IgE-mediated allergic reactions following ingestion [9–11], especially in patients with allergy to peanut [12, 13] and other legume species [14]. Furthermore, inhalation of lupin flour may give rise to asthma in exposed workers [15]. There has been an increase in the use of lupin flour as additive or ingredient in the human food industry paralleling an increase in the prevalence of lupin allergy [16]. The presence of hidden allergens due to contaminations and the use of new potentially allergenic proteins in food products represents a risk for allergic consumers [17]. Therefore, processing methods are needed to minimize the allergenic risk.

Plant protein allergenicity may be variably affected by processing and more specifically by thermal processing, i.e. increased or decreased IgE immunoreactivity [18–27]. Therefore, the protein modifications induced by thermal processing have unpredictable effects on plant allergenic properties. It is known that lupin proteins are rather thermostable [28]. Our previous studies have demonstrated that microwave cooking, boiling and extrusion cooking produced minimal changes on IgE binding to lupin proteins. Boiling in an autoclave at 2.6 bar (138°C) for 20 min drastically reduces or, following longer treatments, even abolishes IgE binding [29], suggesting that lupin allergens are relatively heat stable, and a combination of heat and pressure is required to eliminate their allergenic potency. Instantaneous controlled pressure drop (*détente instantanée contrôlée*; DIC®; patent F2708419, 1995) treatment is a new and highly controlled process used in food technology that combines steam pressure (up to 8 bar) with heat (up to 170°C) for a short time (up to 3 min). The end product is whole seed with a porous texture. The effect of DIC treatment on non-nutritional factors in lupin and other legume seeds showed that this treatment considerably reduced those components without affecting the total protein or lipid content of such legumes [30–33].

Since there is no information yet about the effect of instantaneous controlled pressure drop on legume allergens, the aim of this study is to evaluate the effect of DIC on IgE binding in vitro to lupin seeds.

Materials and Methods

Plant Material

Sweet white lupin seeds (*Lupinus albus* var Multolupa), supplied by the Servicio de Investigaciones Agrarias (Badajoz, Spain), were manually dehulled and the cotyledons were subjected to instantaneous controlled pressure drop.

DIC Treatments

DIC treatment generally consists in placing the moistened product in a processing chamber and exposing it to steam pressure (up to 8 bar) at high temperature (up to 170°C) over a relatively short time (a few seconds to some minutes). The end of the thermal treatment involves an abrupt pressure drop, which is achieved by the instantaneous opening of a valve between the treatment chamber and a vacuum tank (50 mbar) with a volume which is at least fifty times greater than the initial volume. There is a sharp fall in chamber pressure, which leads to a partial vaporization of the water in the products and a modification of the product texture, and results in a porous structure depending on the properties of the product treated and the operating conditions. Thus, DIC re-texturizes biological products, improving their functional properties and makes them easier to use in industrial processes [30–33].

Lupin cotyledons were treated at 3, 4.5 and 6 bar for 1, 2 and 3 min with a constant initial water content of 10.09 g water per 100 g dry matter.

Protein Extracts

Raw and processed seeds were ground to pass through a 1-mm sieve (Tecator, Cyclotec 1093), and the flours were defatted with n-hexane (34 ml/g of flour) for 4 h, shaken and air-dried after filtration. The resulting defatted flours were extracted twice in a solution of 0.05 M Tris-HCl (pH 8.0) buffer with salt (0.5 M NaCl) at a 1:10 w/v ratio for 1 h at 4°C by stirring. The extract was centrifuged at 27,000 g for 20 min at 4°C, and the combined supernatants were dialyzed against distilled water for 48 h at 4°C using dialysis membranes with a cutoff of 3.5 kDa (Spectra/Por®, Serva, Heidelberg, Germany) and freeze-dried [29]. The soluble protein content of all extracts was determined by the Bradford dye-binding method (Bio-Rad, Hercules, Calif., USA) using bovine serum albumin (Sigma, St. Louis, Mo., USA) as a standard. The total nitrogen content was determined using the Kjeldahl procedure [34]. The total protein content was calculated as $N \times 5.45$ [35].

Patient Sera

A serum pool was obtained from 19 patients sensitized to lupin (*L. albus*). All individual sera showed specific IgE levels from 0.38 to 100 kU/l (median = 17.33 kU/l), as quantified by the CAP-fluorescent enzyme immunoassay (FEIA) System (Pharmacia Diagnostic, Uppsala, Sweden).

Electrophoresis and Immunoblotting

SDS-PAGE was performed according to Laemmli [36]. Samples were mixed with XT Sample Loading Buffer (Bio-Rad) and XT Reducing Agent (Bio-Rad), heated at 90°C for 10 min, and electrophoresed in 12% Bis-Tris Criterion XT Precast Gel (Bio-Rad). Proteins were visualized with Coomassie brilliant blue R250 staining.

Table 1. Effect of DIC® treatment on whole extract protein content (g/100 g dry matter) and total crude protein content (g/100 g dry matter) of *L. albus* seeds

Sample	Pressure bar	Time min	Whole extract protein g/100 g dry matter	Total crude protein g/100 g dry matter
Raw	–	–	24.92 ± 1.39 ^a	38.69 ± 0.08 ^a
DIC-1	3	1	7.64 ± 0.55 ^b	40.26 ± 0.00 ^b
DIC-2	6	1	5.11 ± 0.59 ^c	39.96 ± 0.25 ^b
DIC-3	4.5	2	4.69 ± 0.21 ^c	39.55 ± 0.16 ^c
DIC-4	3	3	3.95 ± 0.17 ^c	40.04 ± 0.00 ^b
DIC-5	6	3	3.95 ± 0.54 ^c	40.23 ± 0.08 ^b

^{a-c} Figures (means ± SE; n = 4) followed by different superscripts in the same column were significantly different ($p < 0.05$) compared to Duncan test.

Western blotting was performed by electrophoretic transfer to poly(vinylidene difluoride) membranes (Bio-Rad) at 220 mA during 1.5 h, at room temperature, essentially according to the method of Towbin et al. [37]. The membrane was blocked with phosphate-buffered saline (pH 7.4) with 0.1% Tween 20 containing 5% fat-free milk powder at room temperature and then incubated overnight at room temperature with pooled sera (1:3 dilution). A polyclonal goat anti-human IgE conjugated with alkaline phosphatase (diluted 1:500) was used as second antibody (Caltag, Burlingame, Calif., USA). Following three washings in phosphate-buffered saline with 1% Tween 20, blots were visualized using alkaline phosphatase conjugate substrate kit (Bio-Rad).

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.

Statistical Analysis

All experiments were done in duplicate, and the data were statistically analyzed by multiple regression and ANOVA analysis using the Statgraphic/PC 4.1 software. Comparisons between means were performed by Duncan's test ($p < 0.05$).

Results and Discussion

The values of whole extract and the total crude protein content of raw and processed samples are summarized in table 1. As expected [38], the whole extract protein content of raw samples (24.92 g/100 g dry matter) was lower than the total crude one (38.69 g/100 g dry matter) measured using the Kjeldahl procedure, with the crude protein content being similar to those previously reported for *L. albus* [2, 30]. There was a noticeable reduction in the soluble protein content of all DIC-processed samples,

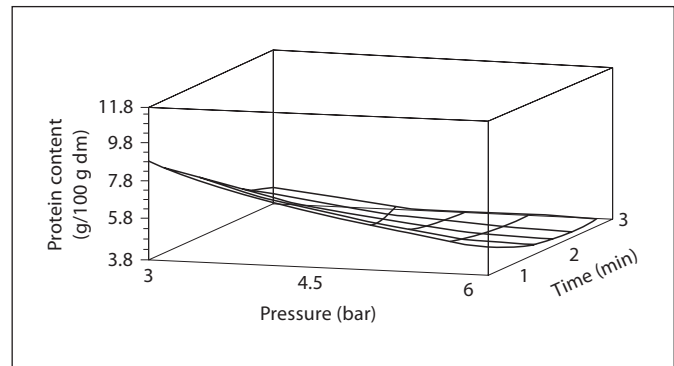


Fig. 1. Response surface representation estimated by multiple regression analysis of protein content (g/100 g dry matter, dm) of lupin seeds versus pressure (3–6 bar) and time (1–3 min) applied using DIC®.

reaching for some severely treated samples up to 81.2% (DIC-4 and DIC-5) compared to raw samples. ANOVA revealed that the decrease in the lupin soluble protein content was significantly associated with time and pressure but not the total protein. The estimated response surface carried out by multiple regression (fig. 1) showed that an increase in steam pressure or treatment time induces a decrease in protein solubility, which could be due to denaturation, aggregation and/or Maillard reactions [39, 40]. However, the decrease in the soluble protein did not result in a decrease in the total protein content. Our data, in agreement with the study by Haddad and Allaf [30], did not show any reduction in the total N content measured using the Kjeldahl procedure between raw and DIC-treated lupin seeds.

The SDS-PAGE patterns of the protein extracts of raw and processed lupin cotyledons are presented in figure 2. Raw lupin cotyledon (lane 1) was composed of numerous bands with molecular weights between 126 and 14 kDa, showing bands corresponding to conglutin β (62–15 kDa), conglutin α (46, 42, 36, 31 and 20 kDa) and conglutin γ (26 and 17 kDa) [41].

Following DIC treatment at 3 bar for 1 (lanes 2 and 3) and 3 min (lanes 6 and 7), only a few bands between 62 and 54 kDa identified as conglutin β disappeared, the 20-kDa conglutin α subunit and those of around 32 and 30 kDa were with lower intensity as treatment time was increased. A major decrease in the number of bands in the range of 66–20 kDa was observed in DIC samples at 6 bar for 1 min (lanes 4 and 5). The disappearance of protein bands was clear at 6 bar for 3 min (lanes 8 and 9). In this sample, only two major bands of around 26 and 29.5 kDa

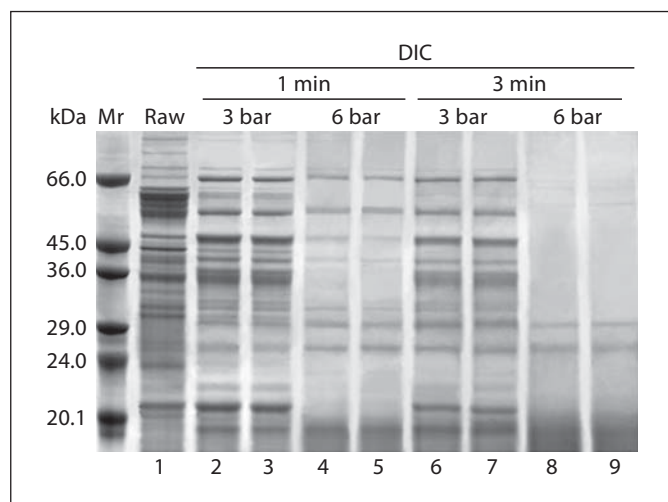


Fig. 2. SDS-PAGE analysis of whole protein extract from raw (lane 1), and DIC-1 (lanes 2 and 3)-, DIC-2 (lanes 4 and 5)-, DIC-4 (lanes 6 and 7)- and DIC-5 (lanes 8 and 9)-processed lupin samples (20 µg protein per lane). Mr = Molecular weight marker.

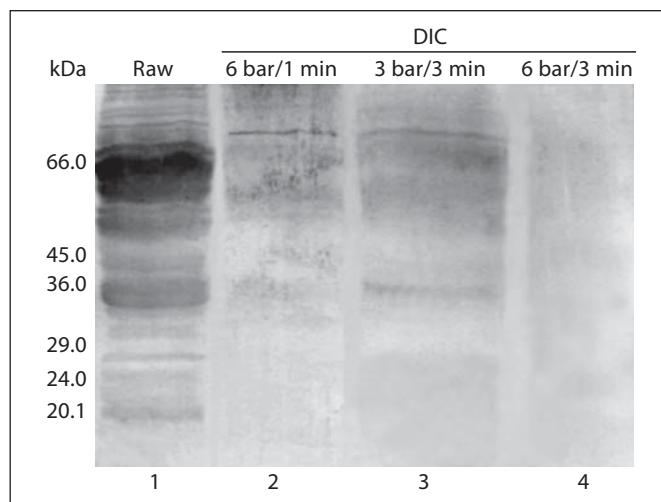


Fig. 3. IgE immunoblotting analysis of whole protein extract from raw (lane 1), and DIC-2 (lane 2)-, DIC-4 (lane 3)- and DIC-5 (lane 4)-processed lupin samples and using a serum pool from 19 patients sensitized to lupin (20 µg protein per lane). The molecular weights (kDa) are given.

and some <20 kDa were detected by SDS-PAGE. Similar protein pattern degradation was found by Alvarez-Alvarez et al. [29] in autoclaved lupin seeds. DIC as well as autoclave at harsh conditions induced the same effect on the protein pattern. The SDS-PAGE protein patterns of lupin seeds treated by DIC at 3 bar for 1 and 3 min were similar to those observed with autoclaved lupin seeds at 1.2 bar for 5 and 20 min, respectively.

In our previous work, two lupin proteins of 20 and 34.5 kDa have been identified as major lupin allergens using 23 patient sera [29]. Other recent studies corroborate that the 20-kDa lupin allergen is the conglutin α basic subunit (legumin-like protein) and demonstrated cross-reactivity with other legumes, e.g. pea, peanut and soybean, in which this protein band is also present [14]. Moneret-Vautrin et al. [13] found an IgE-immunoreactive protein band of 43 kDa. According to Magni et al. [14], a 43-kDa band cross-reacted with peanut allergen (Ara h 3) and coincided with the unreduced conglutin γ [13, 42].

Extractions of total protein with SDS sample buffer were also carried out for raw and DIC-treated lupin flours. Immunoblotting of total and soluble protein fractions did not show differences in IgE-immunoreactive band patterns, and therefore soluble and insoluble proteins showed identical IgE in vitro reactivity. In figure 3, the soluble protein extract was analyzed by IgE immunoblot-

ting. The allergenic pattern of raw sample coincided with that previously reported [29] using other different patient sera, and the same major lupin allergens of 20 and 34.5 kDa could be observed. After DIC treatment, a reduction in the total IgE-reactive bands was detected. The major allergens were still present in these samples treated at 3 bar. However, at 6 bar for 1 min, the 34.5-kDa band was still present, but after 3 min at 6 bar no IgE-immunoreactive band was found in soluble or insoluble protein fractions (data not shown).

Several authors have reported that heat treatment can differently affect legume allergenicity. A decrease in IgE binding in a serum pool from 24 lentil-allergic children was found in boiled lentil extracts, but a strong allergenicity remained [18]. There were no differences between crude and boiled peas in IgE immunodetection in rabbit anti-vicilin serum and in a serum pool from 18 patients with pea allergy [19]. Heating of soybeans by boiling (120 min) or microwave (25 min) treatment decreased allergenicity, with a residual potential risk for some tested allergic individuals [20]. Several authors have indicated increased allergenicity by roasting peanuts [21–24]. Beyer et al. [22] observed that in contrast to roasting, boiling (20 min) and frying (10 min) reduced allergenicity in peanuts. According to Maleki and Hurlburt [24], structural modifications, reduced solubility, resistance of peanut allergens, Ara h 1 and Ara h 2, to digestion, resulting from

roasting, in addition to the effect of the Maillard reaction products could contribute to the enhanced IgE-binding capacity of roasted peanut.

The lupin allergenicity was only slightly affected by boiling, microwave and extrusion-cooking, whereas it could be eliminated by autoclaving at 2.6 bar for 30 min [29]. To date, little is known about the effect of the pressure technique process, i.e. autoclaving and DIC, on allergenic proteins. Venkatachalam et al. [25] and Brenna et al. [26] investigated the effect of autoclaving at 1.03 bar (121 °C) up to 30 min on the antigenicity of almonds (amandin) and peach nectar (Pru p 1) protein, respectively. They observed that these pressure techniques were unable to decrease allergenicity. Malley et al. [27], however, found that autoclaving of green pea at 120 °C for 15 min reduced its allergenicity. A similar reduction has been observed with lupins treated by autoclaving at 121 °C (1.2 bar) for 20 min; treatment at 2.6 bar for 30 min abolished the lupin allergenicity [29]. Compared to autoclaving, DIC at 6 bar eliminated overall lupin in vitro allergenicity but in a shorter processing time (3 min), which represents an advantage for future potential applications in the food industry.

This study corroborates our previous findings about the high thermal resistance of the major lupin allergens [29]. The present results support that lupin seeds treated

with processing technologies combining heat and steam pressure, such as instantaneous controlled pressure drop, could eliminate lupin in vitro allergenicity. Taking into account that IgE in vitro reactivity is an indicator of potential in vivo allergenicity, further in vitro assays and clinical data are required to confirm that DIC treatment can reduce lupin allergenicity. Moreover, labeling of processed and non-processed food products with allergenicity risk should be the first step to protect consumers from food allergy combined with the development of specific assays detecting allergens in complex food matrixes. In the near future, sensory analysis of new food products (e.g. cakes) from processed lupin seeds could be essential for their application in food industry.

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