

IgE binding capacity of apple allergens preserved after high pressure treatment

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(Received 1 September 2009; final version received 1 October 2009)

Two strategies were performed to assay the antigenicity of an apple extract (Golden Delicious) after combined pressure/temperature treatments using pools of sera of patients with positive serum IgE to apple. The IgE binding capacity of SDS denatured proteins was tested after electro-transfer to membranes. Additionally, competitive RAST inhibition tests were carried out with the native apple proteins. Antigenicity was also assessed after 10 months of storage. In general, the results with sera of Spanish patients obtained by the RAST inhibition method confirmed the negligible effects of high pressure treatments up to 800 MPa on apple antigenicity and IgE binding, similarly to other European populations. Immunoblots revealed, contrarily, that heat is more important than pressure in inducing irreversible changes in key epitopes. Furthermore, pressure treatments (up to 800 MPa) preserved inhibition scores in frozen apple extracts closer to the untreated controls during at least 10 months, which could be relevant in the design of strategies to preserve allergen antigenicity.

Keywords: apple allergens; high pressure; Mal d1; RAST; immunoblots; specific sera

1. Introduction

Apple allergy is an IgE-mediated food allergy caused by proteins abundant in the peel. The most important apple allergens have been identified, and cross-reactivity with several allergen families has been described, with birch and components of the Rosaceae family (apple, peach, apricot, etc.). Across Europe, sensitisation profiles determine the clinical expression of apple allergies. In Northern countries, symptoms are mild (predominantly isolated oral symptoms), and mainly related to birch pollinosis. In the Mediterranean countries, however, apple allergy is severe, with systemic reactions related to peach allergy and the predominance of Mal d3 (or LTP protein) [1]. LTP is an extremely stable protein that is resistant to food processing and to gastro-intestinal digestion.

High hydrostatic pressure has specific advantages over other technologies in terms of microbial inactivation and the effects on proteins, enzymes or other food constituents. Pressure is known to inactivate food spoilage microorganisms and pathogens, presenting good perspectives to prolong food shelf-life [2], while the impact on nutrients and healthy compounds are known to be

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minimal, as it is the case of apple or apple products [3]. Furthermore, abundant experimental data demonstrate that pressure is able to originate irreversible changes on the tertiary structure of proteins [4]; thus conformational epitopes might be altered after pressure treatment. However, short-chain peptides are less modified, unless peptidases [5] or *P/T* combinations are used [6]. Primary circular dichroism results were interpreted as a high pressure induced instability of individual apple allergens [7]; however, those results could not be further corroborated by other authors in more recent publications [8,9]. Other recent data illustrate the effects of pressure on the antigenicity of several food products. Those works generally report on the high pressure stability of food allergens. Thus, for example, the highly thermal sensitivity of the main celery allergens (Api g1 and profilin) has been reported by [10], contrary to the pressure (600 MPa, 20 °C) stability of the same molecules. Moreover, Han et al. [11] remarked on the pressure stability but thermal instability of beef allergens studied *in vitro* with immunoblots and ELISA. The preservation of IgE binding of tomato allergens after high pressure treatments was also pointed out [12]. In a different approach, high pressure was found to be a successful technology with which to induce the permeation of rice grains and the extrusion of rice allergens [13], to generate hypoallergenic rice. Those authors also reported on the fair pressure stability of the studied rice allergens.

This work aimed at studying the effects of combined pressure/temperature treatments and a storage period of 10 months on the *in vitro* IgE-binding capacity of an apple extract of Golden Delicious apples. The study was performed with sera of Spanish patients with positive specific serum IgE to apple (*Malus domestica*), and would therefore be complementary to the available data from Northern and Eastern European countries.

2. Materials and methods

2.1. Apple extract

Sodium phosphate buffer 10 mM pH 7 was mixed with 2 mM EDTA in 0.2% PVPP 40,000 and dissolved 1:1 in 2058 g of freshly prepared pureé (with peel, without core) of *Golden Delicious* apples. The mixture was homogenised using a Waring Blender for 1 min at low speed. The extraction took place for 2 h at 10 °C, agitating with a magnetic stirrer. Afterwards samples were centrifuged at 12,000 r.p.m. during 35 min at 8 °C. The final volume recovered after discarding the pellet was 3.35 mL. The supernatant was filtered successively through Whatman (Kent, UK) filter papers, a GFC 1.2 µm and a GFF 0.7 µm. The last step was performed with a 0.45 µm filter from Millipore (Schwalbach, Germany). Samples were dialysed against water overnight with a 3.5 kDa cut-off membrane. The concentration of proteins after the dialysis step was 3.4 µg mL⁻¹.

2.2. Patient sera

Serum samples were obtained from nine patients with positive specific serum IgE to apple (*Malus domestica*), as quantified by using the CAP-FEIA system (Pharmacia Diagnostic, Uppsala, Sweden). All individual sera showed specific IgE levels above 0.35 kU L⁻¹ defined by UniCap, and were positive in skin prick tests.

2.3. High-pressure treatments

Pressure experiments were conducted in a U101 hydraulic press (High Pressure Research Centre, Polish Academy of Science, Warsaw, Poland) for up to 11,000 bar. The U101 is a manually operated twin piston hydraulic press with an automatic piston return (80 mm piston movement;

100 mm piston length) equipped with specialised accessories for pressurising vessels. The vessel is a cylinder made of steel, with an inside diameter of 16 mm and a height of 150 mm. The piston position is monitored with a linear transformer transducer and the pressure-measuring unit is an in-vessel manganin pressure gauge; both are digitally displayed. The transmitting medium was petroleum ether (boiling point: 80–100 °C). The pressure rise required ca. 120 s. Samples were pressurised in Teflon ampoules (10.2 mL) with double closure. The temperature in the vessel was controlled with a Polystat thermostat (Huber, Offenburg, Germany).

2.4. Storage

Pressurised samples were stored at -18°C until IgE binding was measured. Those samples were determined as “fresh extract”. For *in vivo* diagnostics, the activity of allergen solutions should be preserved over longer periods. Thus, a part of the treated samples was also kept frozen for 10 months. These samples were labelled “frozen solution”. Other pressurised samples were freeze-dried and kept dry at -18°C for 10 months and were only measured after storage. Those samples were named “lyophilised extract”.

2.5. SDS-PAGE and immunoblotting

Protein electrophoresis (SDS-Page) was performed according to [14]. Samples were mixed 3:1 with loading buffer (8% SDS, 8 mM EDTA, 40% glycerol, 1 M β -mercaptoethanol and 0.01% bromophenol blue in 0.25 M Tris-HCl pH 7.5) and denatured for 10 min at 100°C . Samples were then separated on 10% analytical SDS-polyacrylamide gels employing a Mini-Protean III apparatus (Bio-Rad), and either stained with Coomassie Brilliant Blue R-250 or electrotransferred to poly(vinylidene difluoride) membranes (Bio-Rad). Samples were blotted by applying a constant current of 250 mA for 2 h at room temperature. Blots were blocked in phosphate-buffered saline plus 0.1% Tween 20 containing 1% fat-free milk powder for 1 h at room temperature, and then incubated at room temperature with pooled sera. A polyclonal goat anti-human IgE conjugated with alkaline phosphatase was used as a second antibody (diluted 1:500) (Caltag, Burlingame, CA). Finally, immunoreactive bands were visualised using the alkaline phosphatase conjugate substrate kit from Bio-Rad. Non-specific binding of the anti-IgE conjugate was measured in a similar blotting procedure, omitting the incubation step with patient sera.

2.6. IgE inhibition assays

RAST: IgE binding to apple allergens was assessed by radioallergoabsorbent test (RAST). A non-treated apple extract was coupled to CNBr-activated discs at an optimal concentration of $10\text{ }\mu\text{g mL}^{-1}$. The conjugated discs were incubated with the pool of sera for 3 h. They were then washed three times with PBS containing Tween 20 at 0.5% (v/v) (Sigma–Aldrich, St. Louis, MO), and finally $50\text{ }\mu\text{L}$ of ^{125}I -labelled anti-human IgE (Pharmacia, Uppsala, Sweden) was added and the mixture incubated overnight. RAST values (% of the label uptake) were assessed using two negative controls. A positive value was considered to be the mean $\pm$ 2 SD of those negative controls. This was 1% of the label uptake. All samples were tested in duplicate and in the same assay. The total sera IgE was assessed by IMx-method (Abbott Diagnostics, Chicago, IL).

RAST inhibition: The IgE binding potential of treated and non-treated apple extracts was studied by competitive RAST-inhibition. Each serum ($50\text{ }\mu\text{L}$) was co-incubated overnight with $30\text{ }\mu\text{L}$ of inhibitor (apple extracts, treated and non-treated) at a final concentration of $1\text{ }\mu\text{g mL}^{-1}$, and afterwards conjugated discs were added. The rest of the procedure was as for the RAST assay.

2.7. Statistics

Software JMP® Version 3.1.6.2 (SAS Institute, NC) was used for statistical analysis.

3. Results

3.1. SDS-PAGE and immunoblotting of an apple extract

Denaturing SDS-PAGE was performed in order to study the effects of temperature or pressure/temperature combinations on the protein pattern. After treatments, 20 µg of total protein was run in each well and stained with Brilliant Blue; a similar pattern of proteins was observed for all samples (Figure 1, I). Furthermore, the protein major bands were well preserved among treatments. However, a certain effect of the treatments was observed in the protein pattern, since the intensity of bands below 22 kDa of heated (60 °C) and high pressure treated (400/600/800 MPa at 60 °C) samples decreased remarkably, if compared with the untreated control.

The IgE binding capacity of the treated apple proteins was assayed by immunoblotting using a pool of patient sera with specific IgE. Three major bands, weighing over 36 kDa, around 23 and over 16 kDa were identified in the untreated samples. The highly reactive band over 36 kDa corresponds probably to a glycoprotein commonly identified in apple allergic patients [15]. The estimated molecular weight of the other two bands likely indicates the well characterised Mal d2 and Mal d1, respectively. The dialysis membrane had a small cut-off, but the low molecular weight LTP protein (Mal d3) was, however, not identified with this technique, although it is expected to cause a remarkable amount of systemic reactions in Spanish allergic patients. Apple extracts that

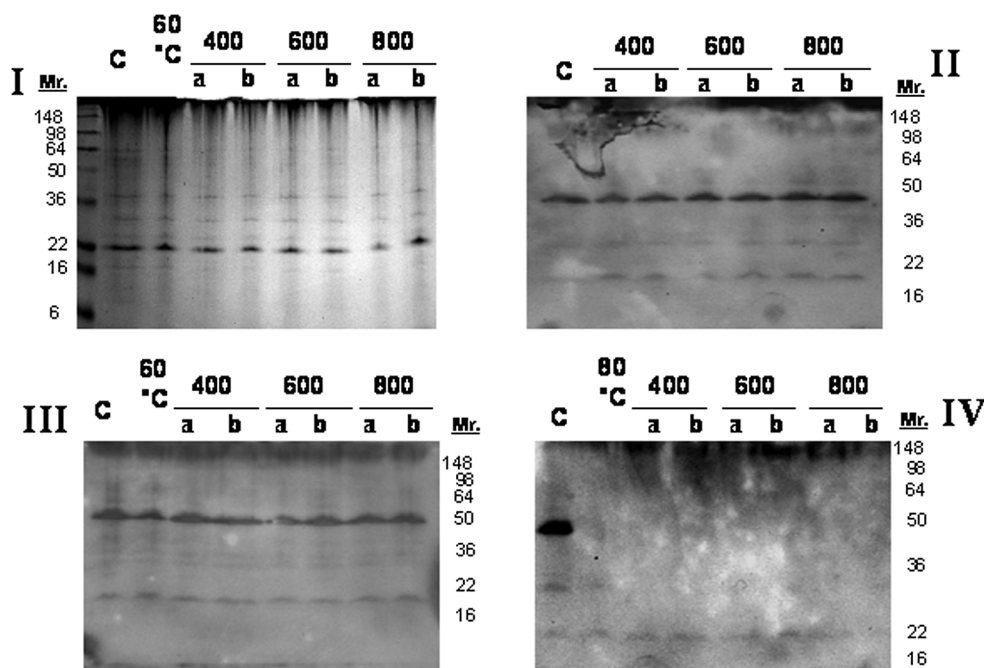


Figure 1. Immunoblots of apple extracts with a pool of sera of allergic patients. All sera contained specific IgE against apple determined by the CAP system, with values ranging from Class 4 to 5, without specific antibodies to *Betula verrucosa*. (C) Untreated extract. (I) SDS-PAGE of samples at 60 °C Coomassie stained; (II) samples at room temperature; (III) samples at 60 °C for 10 min; (IV) samples at 80 °C for 10 min. (a) and (b) represent duplicates of each condition.

were high pressure-treated at room temperature (Figure 1, II) bound IgE antibodies in a pattern comparable to that of the untreated extract; so pressurisation alone has not led to a visible effect on the IgE binding capacity of apple protein epitopes. Full preservation of allergens was also evident after pressure treatment at 60 °C (Figure 1, III). However, after treatment at 80 °C for 10 min, only the band around 20 kDa was detectable (Figure 1, IV). Thus, surprisingly, and despite the strong denaturation before SDS-Page, the remarkable loss in IgE binding capacity of samples treated at 80 °C could be mainly attributed to heat. Pressurisation combined with heat treatment at 80 °C promoted only a stronger reduction in IgE binding at 800 MPa, since bound IgE almost disappeared after blotting. Such a decrease in the antigenicity of apple extracts induced by high temperatures has also been observed by other authors on immunoblots [16], and occurs most probably due to the destruction of conformational epitopes affecting heat-sensitive allergens.

The minimal effects of pressure on IgE binding could be attributed to the markedly strong pressure stability of alpha-helices and beta-sheets, which are related to the protein spatial configuration and are key structures for IgE binding to epitopes. Apple allergens are in general described as pressure stable, and only treatments at high temperatures are able to promote a remarkable reduction on IgE binding. The more stable allergen detected at approx. 20 kDa is likely to be Mal d1, which has been reported to resist high pressure and temperature combinations without losing the alpha-helix content [8,9].

3.2. RAST inhibition

Results of RAST inhibition allowed a quantification of the IgE binding capacity of proteins in the native state. RAST inhibition was performed with allergic patient sera showing cross-reactivity to birch pollen allergens, in order to amplify the IgE binding capacity of treated apple extracts. CNBr-activated discs were conjugated with fresh non-treated apple extracts and afterwards incubated with the pool of sera of several patients of known allergen specificity, with the addition of 1 or 10 $\mu\text{g mL}^{-1}$ of each treated sample as the inhibitor. Distribution of frequencies of inhibition ranged from 10 to 40 at $\mu\text{g mL}^{-1}$, while at 10 $\mu\text{g mL}^{-1}$ values occurred between 70 and 90 (Figure 2). Results indicate that IgE binding capacity is dependent on protein loads. Inhibition

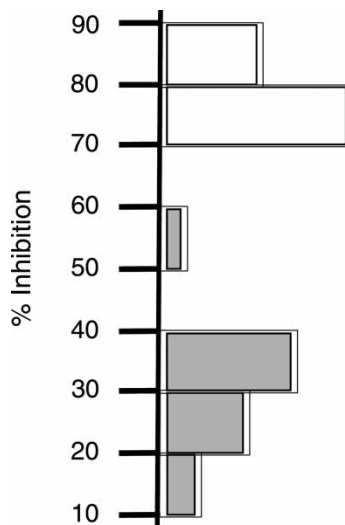


Figure 2. Frequency distribution of RAST inhibition values with fresh extracts at 1 or 10 $\mu\text{g/mL}$. White and grey bars represent inhibition with 10 and 1 $\mu\text{g/mL}$, respectively.

scores with $10 \mu\text{g mL}^{-1}$ were close to saturation. Incubation with $1 \mu\text{g mL}^{-1}$ as a non-saturating condition was then chosen for further experiments.

Fresh extracts, lyophilised extracts and frozen solutions were tested using the RAST inhibition method in order to evaluate the effects of sample preparation on allergen conservation. Results are summarised on Table 1. Inhibition potential of fresh and lyophilised extracts preserved at -18°C during 10 months was not significantly different ($P > 0.05$). Frozen extracts kept for 10 months at -18°C were, however, significantly different from the others, showing a remarkably lower IgE binding capacity, probably due to active proteolytic enzymes and subsequent activation during thawing, or a less probable allergen degradation during storage [17].

Complete data corresponding to the *T* and *P/T* treated fresh extracts, lyophilised extracts and frozen solutions are represented in Figure 3. The scores in fresh and lyophilised groups clustered tightly around the mean score, but frozen data varied, indicating weak inhibition and a big dispersion, probably related to allergen degradation in the last group. Generally, the fresh and lyophilised extracts promoted the best performance. Samples kept frozen for 10 months showed the weakest IgE binding capability.

Detailed data on RAST inhibition scores for *T* and *P/T* samples are recorded in Figures 4 and 5. The decrease in IgE binding capacity of samples treated at 80°C found on immunoblots was not reflected in the total IgE binding capacity of the apple extracts tested by RAST. In general, the applied pressure or temperature treatments have not diminished the total antigenicity of the

Table 1. Student's *t*-test analysis of the mean values of the RAST inhibition scores with fresh apple (a), lyophilised (10 months) (b) and frozen (10 months) (c) extracts.

Sample preparation	Mean	SD	<i>t</i> -Test	DF	Probability
A = Fresh extract	31.63	9.01	A vs. B	44	0.1779
B = Lyophilised extract	35.75	11.27	B vs. C	44	<0.0001
C = Frozen solution	6.82	18.67	C vs. A	44	<0.0001

Note: Confidence interval was set at 95%.

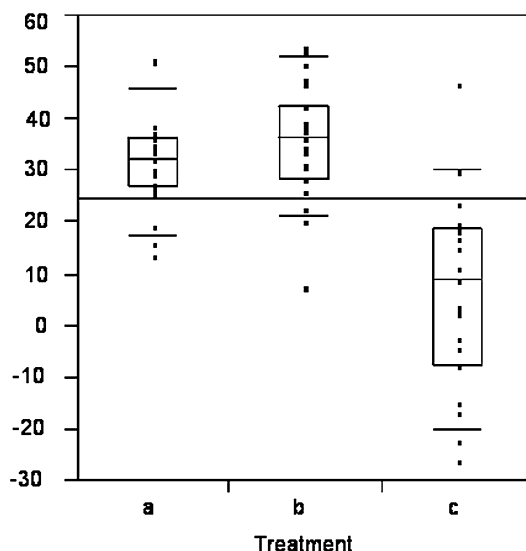


Figure 3. RAST inhibition scores with fresh apple (a), dried (10 months) (b) and frozen (10 months) (c) extracts. Arrows signify values of inhibition with non-treated samples. Boxes include the following: extern limits are 90–10th quantiles; intern limits are 75–25th quantiles; the middle line corresponds to the group mean; the crossing line corresponds to the sample mean.

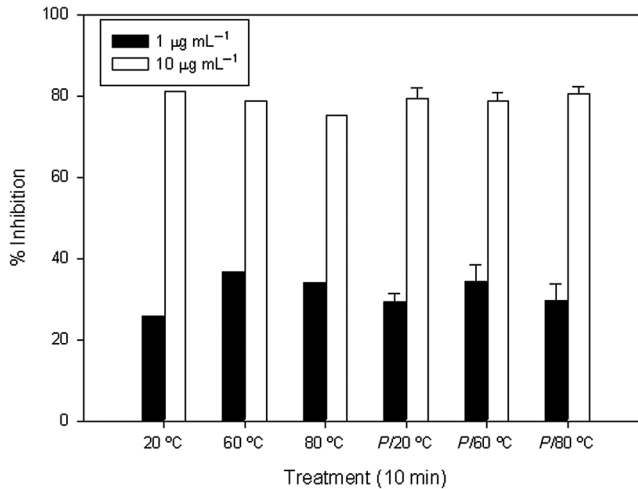


Figure 4. RAST inhibition scores with fresh apple extracts. *P* is an average of scores obtained at 400, 600, and 800 MPa.

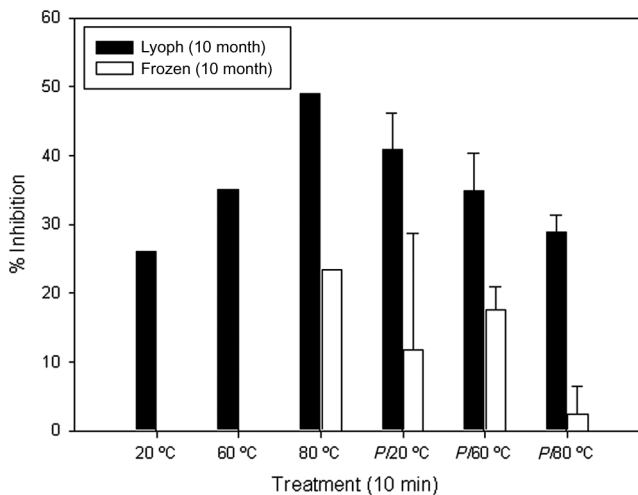


Figure 5. RAST inhibition scores with lyophilised and frozen apple extracts kept at -18°C for 10 months. *P* is an average of the scores obtained at 400, 600, and 800 MPa.

native protein extracts. Indeed, over 75% of all *P/T* treated samples showed slightly higher RAST inhibition scores than the untreated controls. Furthermore, differences among the 400, 600, and 800 MPa treatments were not statistically significant ($P > 0.05$) (seeking simplicity, those data were represented together). Among the evaluated treatments, pressurisation (400, 600, 800 MPa for 10 min) at room temperature preserved inhibition scores in frozen apple extracts closer to the untreated controls during at least 10 months of storage. Those treatments had an influence on allergen stability, probably related to the inactivation of proteolytic enzymes.

Other authors have generally reported very low effects of pressure on *in vitro* antigenicity of different foods. But Han et al. [11] could corroborate the effects observed in immunoblots after heating beef extracts at 100°C with ELISA inhibition scores. However, in this work, a discrepancy could be found between RAST and immunoblots at 80°C . The extreme pressure and temperature resistance of Mal d1 might have been responsible for the high inhibition scores recorded after all treatments. The stability of apple allergens was also demonstrated in several works [8,9];

Mal d1 stability itself might explain the discrepancy between immunoblots and RAST assays [18]. Following those authors, a correspondence between RAST inhibition scores and results of prick-to-prick tests could also be inferred; therefore, extracts treated with pressure up to 800 MPa and/or temperature up to 80 °C would probably present allergenic properties in *in vivo* tests.

Acknowledgements

Authors thank the Spanish Comisión Interministerial de Ciencia y Tecnología for financial support under grant AGL07-65936-C02. Authors are indebted to the staff at the Servicio de Alergia from Hospital La Paz (Madrid) and especially to Dr Miguel Blanca and Dr Javier Álvarez for technical assistance and very helpful discussions.

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