RESEARCH ARTICLE

High pressure, thermal and pulsed electric-field-induced structural changes in selected food allergens

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Scope: The effects of high-pressure/temperature treatment and pulsed electric field treatment on native peanut Ara h 2, 6 and apple Mal d 3 and Mal d 1b prepared by heterologous expression were examined.

Methods and results: Changes in secondary structure and aggregation state of the treated proteins were characterized by circular dichroism spectroscopy and gel-filtration chromatography. Pulsed electric field treatment did not induce any significant changes in the structure of any of the allergens. High-pressure/temperature at 20°C did not change the structure of the Ara h 2, 6 or Mal d 3 and resulted in only minor changes in structure of Mal d 1b. Ara h 2, 6 was stable to HPP at 80°C, whereas changes in circular dichroism spectra were observed for both apple allergens. However, these changes were attributable to aggregation and adiabatic heating during HPP. An ELISA assay of temperature treated Mal d 3 showed the antibody reactivity correlated well with the loss of structure.

Conclusion: In conclusion, novel-processing techniques had little effect on purified allergen structure. Further studies will demonstrate if these stability properties are retained in food-matrices.

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1 Introduction

High-pressure (HP) and pulsed electric field (PEF) treatments are novel-processing techniques which have the potential to alleviate the need for thermal processing of foods. High pressure (400–700 MPa) combined with temperatures around room temperature (5–40°C) and PEF treatments (20–40 kV/cm, and total energy input of 40–1000 kJ/kg) can pasteurize foods while high pressure combined with high temperature (>80°C and >600 MPa) allows sterilization of food. These treatments offer an alternative to high-temperature pasteurization, or chemical preservation with less destruction of factors associated with fresh-like character in foods. In addition to serving as an alternative method of preventing food spoiling, high-pressure and PEF processing can yield physically desirable characteristics in foods after treatment. For example, high pressure and temperature processing has been used in the manufacture of rice-based products such as cooked rice, rice crackers and rice cakes [1, 2]. PEF involves the application of high-voltage pulses (electric field strength of 0.1–40 kV/cm, and total energy input of 0.5–1000 kJ/kg) to electroconductive foods which are placed between the electrodes. The resultant electric field induces movements of ions and permeabilization

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Abbreviations: CD, circular dichroism; **HP,** high pressure; **HRP,** horseradish peroxidise; **LTP,** lipid transfer protein; **PBST,** PBS containing 0.05% v/v Tween 20; **PEF,** pulsed electric field

of cell membranes called electroporation. This biological effect is responsible for a wide range of applications, such as microbial inactivation [3], enhanced extraction of plant material and improved mass transfer processes [4] or stress induction in plant cells [5]. Some of the advantages of PEF treatment over traditional processing are a very short time (milliseconds to microseconds) of treatment and continuous operability.

It is known that secondary and tertiary structure of food allergens is crucial to their allergenic potential. The way in which allergens are folded potentially affects digestion in the gastrointestinal tract [6] and therefore the presence of allergenic peptides in the gut and recognition by serum IgE and hence elicitation of allergic reactions in sensitized individuals. Both HP and PEF treatments have the potential to alter the structure of proteins and hence may alter the extent to which allergens elicit a response. Novel-processing techniques able to reduce allergenicity of foods and even render them harmless would be of considerable benefit to consumers. High-pressure treatment of proteins has been described as having denaturing effects [7] can change emulsifying properties [8] and has the potential to be used for protein renaturation under certain conditions [9]. Studies of the effect of PEF on protein structure are limited in number [10], although PEF treatments of soybean protein isolates [11] purified horseradish peroxidase and pectin esterase [12] have shown interesting effects on protein denaturation, aggregation and structure depending on the protein examined. In the case of horseradish peroxidase and pectin esterase, the authors observed loss of enzyme activity which they attributed to conformational change, although the contribution from indirect effects (*e.g.* associated Ohmic heating) should not be ruled out in these studies.

In this study, we present data relating to the effects temperature, high-pressure and PEF treatments had on three selected, model purified plant allergens: Ara h 2,6 (peanut 2S albumins), apple Mal d 1 and Mal d 3. The peanut 2S albumins, consisting of Ara h 2 and Ara h 6 proteins, are members of the prolamin group of plant allergens [13]. Ara h 2 is generally considered the more problematic allergen despite sharing 59% sequence identity and a high degree of structural conservation with Ara h 6 [14]. Cross-reaction of serum IgE from sensitized individuals with Ara h 2 and Ara h 6 has also been observed [15]. Apple is one of the most common allergenic foods in Europe and contains two predominant allergens. Apple Mal d 3, a nonspecific lipid transfer protein (LTP) and a member of the prolamin family [16], is a problematic allergen in Southern European populations [17]. The apple Bet v 1 homologue Mal d 1 is a major allergen in Northern European populations and is implicated in pollen-associated fruit allergy [17]. Mal d 1 represents a large gene family of Bet v 1-related plant proteins, the major expressed fruit form of the protein being Mal d 1b [18]. The Bet v 1 homologues are PR-10 proteins, thought to be involved in stress and disease response, though their exact function remains unclear.

Novel-processing techniques, such as high pressure and PEF, have focussed on enzyme studies, as examples of proteins. The aim of the study is to assess the impact that novel-processing techniques have on the structure of known allergens, and is of interest to patients that need to eliminate foods containing allergens. The current recommended strategy for allergy sufferers is avoidance, which can be difficult for allergy sufferers to manage, given the nature of labelling and potential changes to or addition of ingredients by manufacturers within prepared food.

2 Materials and methods

2.1 Preparation of recombinant Mal d 1b

The Mal d 1b gene (clone CAD32318) is the major expressed form in apple fruit [18]. The clone was optimised for *Escherichia coli* codon usage and prepared by Geneart AG (Regensburg, Germany). Restriction sites were introduced into the Mal d 1 sequence and used to clone CAD32318 into pET15b upstream of the His-tag coding sequence, so no His-tag would be introduced into the Mal d 1 sequence. The resulting plasmid, pPJ01, was transformed into *E. coli* BL21 (DE3) and cells grown to an A^{595} of 0.6 at 37°C before induction of recombinant protein expression with 0.7 mM isopropyl-β-D-1-thiogalactopyranoside. Cells were incubated with shaking at 37°C for a further 3 h before harvesting by centrifugation and resuspension in lysis buffer (20 mM phosphate, 4 mM EDTA and Sigma Complete protease inhibitors (Sigma, Poole, UK)).

Cell preparations were lysed by three passes through a French Pressure Cell Press (American Instrument, Maryland, MD, USA) (7 MPa). After the removal of cell debris by centrifugation at 18 000 × *g* (30 min) the lysed cell preparation was made up to 50 mL with lysis buffer and ammonium sulphate was added to 65% saturation at 4°C. After stirring for 1 h at 4°C, the solution was centrifuged for 30 min at 18 000 × *g* to remove insoluble proteins and the supernatant loaded in batches of 10 mL onto an FPLC column packed with phenyl sepharose HP6 (high substitution) (Pharmacia, UK) (16 × 120 mm^2) pre-equilibrated in 20 mM phosphate, pH 7.0, 3 M $(NH_4)_2SO_4$. Chromatography was performed using a BioCad Sprint chromatography system (Perkin Elmer, Macclesfield, UK). After washing with 40 mL of mM phosphate, pH 7.0, 3 M $(NH_4)_2SO_4$, proteins were eluted using a linear gradient into 20 mM phosphate, pH 7.0, at 3 mL/min over 100 mL and monitored using absorbance at 220 and 280 nm. Fractions containing Mal d 1b as assessed by SDS-PAGE were pooled and subjected to gel-filtration on a Superdex-75 column (bed dimension = 1.6 × 60 cm^2, GE Healthcare, Little Chalfont, UK) equilibrated in 20 mM phosphate, pH 7.0, 150 mM NaCl at 1 mL/min. Fractions containing proteins of a mass consistent with Mal d 1b (Mr = 15–20 kDa) by SDS-PAGE analysis were again pooled and dialysed against 20 mM phosphate pH 7.0 overnight at 4°C with three buffer changes to remove salt. Concentration was performed using an Amicon 8200 stirred cell equipped with a 1000 Da

membrane (YM1) operated at 20 psi. Protein aliquots were stored at −80°C prior to further use. Protein identity was verified by tryptic digest and MALDI-MS (data not shown).

2.2 Purification of Mal d 3 from apple peel

Purification was carried out according to [19]. Protein was frozen at −80°C prior to further use. Protein identity was verified by tryptic digest and MALDI-MS (data not shown).

2.3 Purification of Peanut Ara h 2, 6 proteins

Purification of peanut Ara h 2, 6 (peanut 2S proteins) was carried out essentially according to the method of [20], with the following modifications. A lower ratio of 25 g defatted flour in 500 mL Tris/HCl buffer was used in the initial extraction phase. Only the Superdex-75 and HQ-Poros (ion exchange) chromatography steps were used, the RP-HPLC step was omitted. The peanut 2S protein fractions were found to contain Ara h 2 and Ara h 6 subunits consistent with those found previously [20]. Identities were verified by tryptic digest and MALDI-MS (data not shown). For brevity, the resultant mixture of allergens Ara h 2 and Ara h 6 is herein referred to as Ara h 2,6.

2.4 Heat treatment at ambient pressure

Protein solutions (1 mL of 1 mg/mL in 20 mM phosphate buffer pH 7.0) were heated treated in sealed 1.5 mL glass vials (Agilent Technologies, Belgium) in a circulating oil bath for 10 min at 20, 60, 80, 90, 100, 110 and 120°C. After heat treatment, samples were removed immediately and transferred to an ice bath to stop any further denaturation. Finally, samples were frozen and stored at −20°C before analysis.

2.5 High-pressure treatment of allergens at ambient temperature

High-pressure experiments were performed in a laboratory scale multivessel high-pressure equipment (custom-made, Resato, Roden, The Netherlands), consisting of six individual vessels surrounded by an isolated heating coil, connected to a cryostat. Protein samples (250–500 μL) of 1 mg/mL for Ara h 2, 6, 0.5 mg/mL for Mal d 1b and Mal d 3 in 20 mM phosphate buffer pH 7.0 were sealed in 500 μL flexible tubes (Elkay, Belgium) and secured in the pressure vessels equilibrated at 20°C in exactly 1 min by tying them to the thermocouples attached to the pressure vessel stoppers. Pressure was built up slowly (100 MPa/min) to allow a maximal dissipation of adiabatic heat. After attaining the desired pressure, the individual vessels were isolated and finally the central circuit was decompressed. The vessels were decompressed after 10 min of pressure treatment. In these experiments, a pressure range of 150–800 MPa was used. The temperature and pressure profile during the treatment was logged. After pressure release, samples were removed in exactly 1 min and cooled in ice-water to stop any further reactions and frozen before analysis.

2.6 High-pressure treatment of allergens at elevated temperature

Protein solutions were pressure treated at 80°C under semi-constant high-pressure and -temperature conditions following the procedure described by De Roeck *et al.* [21]. Briefly, protein samples (1 mg/mL for Ara h 2, 6, 0.5 mg/mL for Mal d 1b and Mal d 3) of 250–500 μL were placed in 500 μL flexible tubes (Rotilabo, Roth, Germany) in a water-filled sample holder at room temperature. The sample holder was transferred to the pressure vessel pre-set at the desired process temperature (80°C). The temperature in the sample holder was allowed to rise to an initial temperature which was dependent on the desired process temperature after pressure build-up and the pressure level. Preheating was the result of heat transfer from the pressure medium to the samples. After achieving the initial temperature necessary to attain the desired process temperature, pressure build-up (10 MPa/s) was started. Pressure and temperature were logged. Final pressure was held for 10 min. Samples were removed exactly 1 min after the pressure was released and transferred to an ice-bath immediately after treatment, and frozen for storage.

2.7 PEF treatment of allergens

PEF processing conditions of low and high intensities have been performed in a Micro batch PEF system designed and constructed at Berlin University of Technology [22]. PEF treatments of Ara h 2,6 (1 mg/mL), apple Mal d 1 (0.5 mg/mL) and apple Mal d 3 (0.5 mg/mL) were carried out in micro cuvettes with aluminium parallel plate electrodes, 20 × 2 mm, 2 mm gap and a volume of 400 μL (Eppendorf, Hamburg, Germany), with various processing conditions as summarized in Table 1. Electric field strengths from 0 to 35 kV/cm and specific energy inputs from 0 to 130 kJ/kg were applied. To minimize temperature increase due to Ohmic heating, throughout the treatment, a frequency of 2 Hz was chosen. Samples were removed from treatment chamber immediately after treatment and frozen for storage at −80°C.

2.8 Analysis of protein secondary structure and aggregation

Changes in the secondary structure of the allergens were assessed using circular dichroism (CD) spectroscopy and

Table 1. PEF processing conditions used for experimental set up

Electric field strength (kV/cm)	Number of pulses	Frequency (Hz)	Total energy input (kJ/kg)	Time (s)	Temperature increase (°C)
Peanut Ara h 2,6					
0-Probe	0	0	0	25	0
5	50	2	1.49	25	0.5
15	50	2	14.2	25	3.3
25	50	2	31.9	25	7.6
35	50	2	60.4	25	14
35	100	2	122	50	29
Apple Mal d3					
0-Probe	0	0	0	25	0
5	50	2	1.21	25	0.29
15	50	2	11.5	25	2.7
19	50	2	18.4	25	4.4
25	50	2	34.5	25	8.2
35	50	2	64.6	25	15
35	100	2	129	50	31

aggregation state monitored using size exclusion chromatography. Far-UV (190–260 nm) CD spectra were recorded at 20°C using a J-710 spectropolarimeter (Jasco, Japan) using 0.5 mm path length cell. Spectra were the average of four accumulations at 100 nm/min with a 2 s time constant, 0.5 nm resolution and sensitivity of $\pm$100 mdeg. Measurements were performed on protein at 0.3–1.0 mg/mL (estimated by Bradford assay) in 20 mM phosphate buffer, pH 7.0. Samples with visible precipitate were not used for CD analysis. Protein samples were heated while CD measurements were taken *in situ* using a water bath connected to the CD instrument.

Size exclusion chromatography was performed using a Superdex-75 column (bed dimension = 16 mm diameter $\times$ 600 mm long, GE Healthcare) attached to an Åkta Basic FPLC system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) with auto-injection by Gilson 234 auto-injector. The flow rate was 1.0 mL/min and the total run length was 120 min in 20 mM Tris/Cl^- pH 8.0 200 mM NaCl. The sample was 50 µL of a 0.3–1.0 mg/mL protein sample in 20 mM phosphate, pH 7.0. Protein elution was monitored using absorbance at 220 and 280 nm. Calibration of retention times *versus* molecular weights was performed using Bio-Rad molecular weight gel-filtration standard (Bio-Rad cat no. 151–1901) (Bio-Rad, Hertfordshire, UK).

2.9 Protein content

Protein concentration was measured using a commercial Bradford assay, (Per-Bio science, Cramlington, Northumberland, UK) using bovine serum albumin as a standard.

2.10 Preparation of Mal d 3 antibodies

A solution of Mal d 3, (0.25 mL of 0.4 mg/mL in sterile saline solution, 0.85% NaCl) was emulsified with 0.5 mL of TiterMax Gold adjuvant (Sigma, Dorset, UK) by repeatedly passing the mixture through a narrow aperture between syringes. Half the emulsified protein was used to inoculate each of the two male rabbits (New Zealand White). The inoculations were repeated twice at monthly intervals after the initial inoculation. Blood was collected 15 days after the final booster injection from ear bleeds into heparinized tubes and centrifuged at $720 \times g$ for 15 min at room temperature to remove cells. The serum fraction was then removed and stored frozen in aliquots at −20°C. Serum from the animal that gave the highest titre against Mal d 3 was selected for further work in the study.

2.11 Analysis of immunoreactivity of Mal d 3

Micro-titration plates (NUNC Maxisorb 96 well, Sigma) were coated by incubation with 0.2 µg/mL Mal d 3 in PBS (0.0015 M KH_2PO_4, 0.008 M Na_2HPO_4, 0.0027 M KCl, 0.137 M NaCl, pH7.4) overnight at 2°C. Plates were then washed five times using PBS containing 0.05% v/v Tween 20 (PBST). For the assay, 100 µL/well of 1/10 000 v/v anti-Mal d 3 antibody was added to 100 µL/well of treated Mal d 3 sample solution. The plate was incubated for 2 h and then washed five times with PBST, before 200 µL of 1/1000 v/v dilution of goat anti-rabbit horseradish peroxidise conjugate (Sigma) was added to each well. The plate was incubated at room temperature for 2 h and then washed with PBST five times. Totally, 200 µL of 0.4 mg/mL *o*-phenylenediamine dihydrochloride (Sigma) in 0.05 M phosphate-citrate buffer containing 0.03% sodium perborate

(Sigma), was added *per* well and plates were incubated in the dark, at room temperature for 30 min. The reaction was stopped by the addition of 50 µL *per* well of 3 M HCl and the absorbance ($\lambda = 492$ nm) of each well was read with an ELISA plate reader (BioRad Microplate spectrophotometer, Bio-Rad). The samples were assayed in triplicate and a calibration curve of 3.8 ng/mL–25 µg/mL Mal d 3 was included on each plate allowing changes in immunoreactivity of the treated Mal d 3 samples to be determined.

3 Results and discussion

3.1 Ara h 2,6

Peanut 2S albumin comprises a mixture of Ara h 2 and Ara h 6, the CD analysis of the mixture showed a spectrum typical of α-helix-rich protein. This is in agreement with the 5-helix superstructure reported for Ara h 6 by Lehmann *et al.* [14] and spectra are consistent with those previously published [20].

No changes in secondary structure were observed following high-pressure treatment at 20 (Fig. 1A) or 80°C (Fig. 1B). As HP treatment often involves a degree of thermal processing due to adiabatic heating, we have attempted to evaluate the possible synergistic or antagonistic effect of high pressure by including samples that were thermally treated only. Thus, Ara h 2,6 was heat-treated at temperatures varying from 60 to 120°C for 10 min before cooling on ice. Figure 1C shows the CD spectra of each of the allergens after thermal treatment. As the procedure involved measurement of CD after cooling of the samples, any changes observed in secondary structure represented the inability of the protein structure to refold after heating.

Ara h 2,6 underwent a transition from α-helix to random coil when heated to 110°C or over (Fig. 1C). This is in partial agreement with the previous studies [14], which showed that recombinant Ara h 2 was stable when heated to 90°C, but the protein lost α-helix when reduced with 1,4-dithiothreitol and then heated. Analysis by gel-filtration (Fig. 2) revealed that the Ara h 2,6 preparation consisted of protein monomers of Mr = 14–17 kDa, and that heating above 100°C induced formation of dimeric (Mr = 26–29 kDa) and tetrameric (Mr = 60–65 kDa) species. At 120°C, dimeric Ara h 2,6 formed 78% of total detectable protein, with tetrameric species forming 5% and monomers the remainder.

In contrast, PEF treatments from 0 to 35 kV did not appear to affect the secondary structure of the Ara h 2,6 proteins, as shown in Fig. 3. These results are in agreement with the previously published data showing no effect of PEF on the secondary structure of proteins [23].

3.2 Mal d 3

Untreated Mal d 3 (Fig. 4) had a CD spectrum similar to those reported previously [19]. No changes in secondary structure were observed following high-pressure treatment at 20°C (Fig. 4A). However, HPP treatment at 80°C (Fig. 4B) caused significant unfolding of α-helix to random coil at higher pressures, especially above 400 MPa, although monitoring of temperature during HPP treatment revealed these changes to be due to adiabatic heating and were not dependent upon pressure (data not shown). Mal d 3 showed a marked loss of structure, from α-helix rich to random coil when heated to 90°C or above (Fig. 4C) at ambient pressure. This is also in agreement with the previous studies of

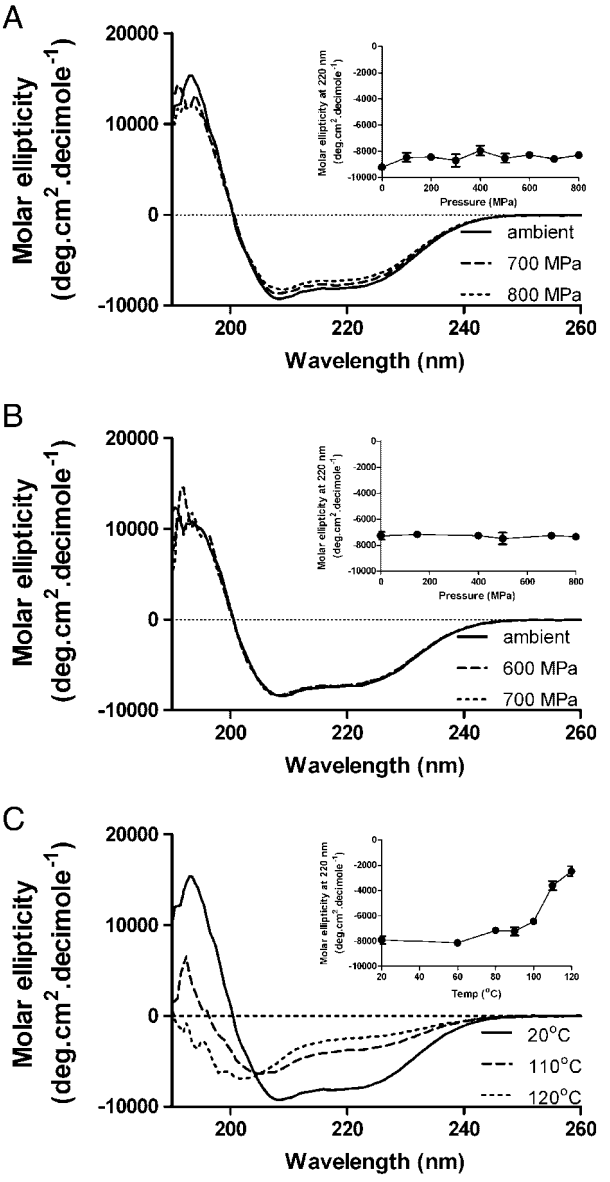

Figure 1. CD Spectra of Ara h 2,6 after treatment at various pressures at 20°C (A), 80°C (B) and after heating to 20–120°C for 10 min and cooling (C). Only three spectra are presented for clarity. Graph insets represent molar ellipticity at 220 nm as a measure of change in secondary structure (mean and standard deviation from three individually treated protein samples).

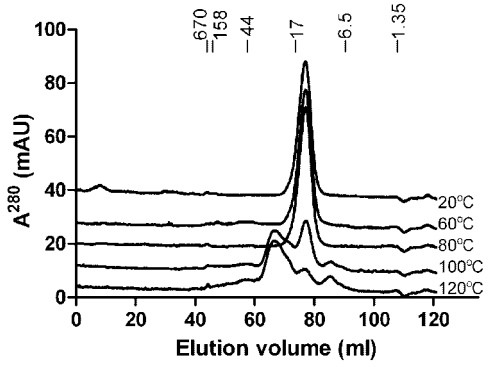

Figure 2. Gel-filtration chromatography of heated peanut Ara h 2,6. Samples of Ara h 2,6 protein were analysed by Superdex-75 chromatography. Masses of calibrants used are indicated at the top of the graph.

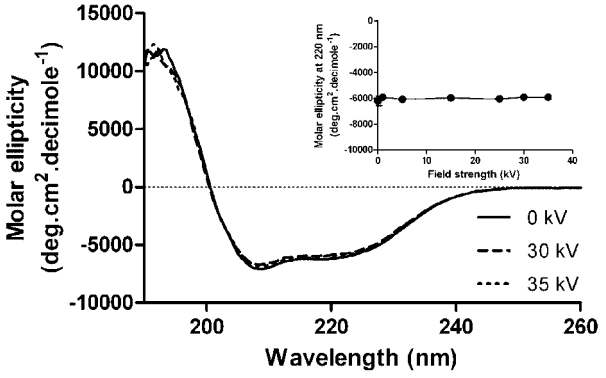

Figure 3. Effect of PEF on the secondary structure of peanut Ara h 2,6 protein as determined by CD spectroscopy. Only three spectra are presented for clarity. Graph inset represents molar ellipticity at 220 nm as a measure of secondary structure (mean and standard deviation from three individually treated protein samples).

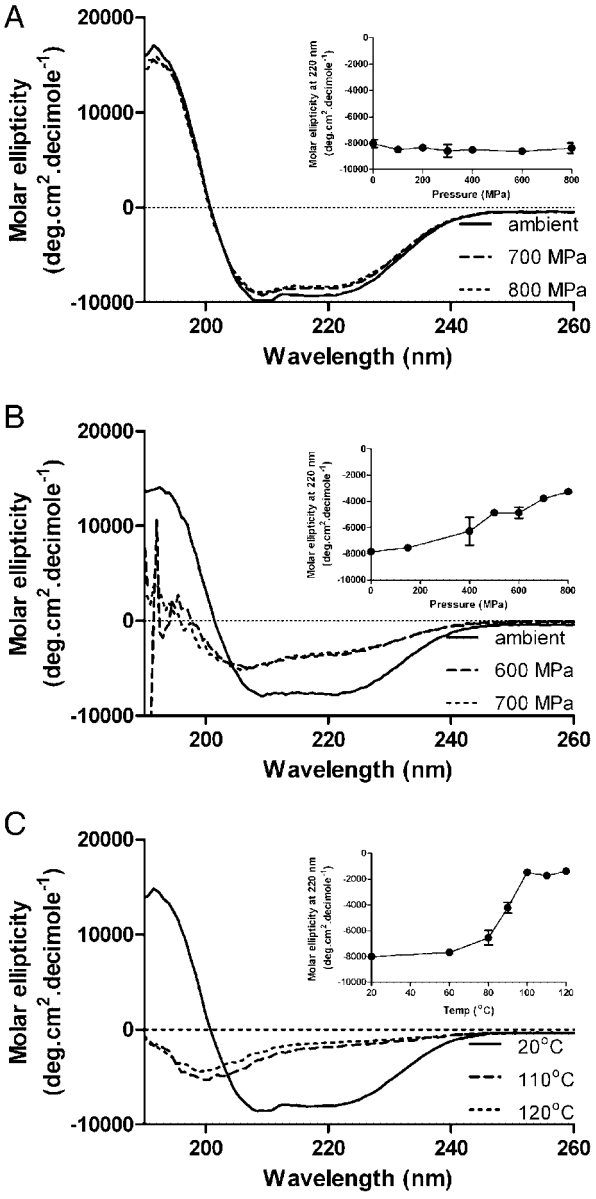

Figure 4. CD Spectra of Mal d 3 after treatment at various pressures at 20°C (A), 80°C (B) and after heating to 20–120°C for 10 min and cooling (C). Only three spectra are presented for clarity. Graph insets represent molar ellipticity at 220 nm as a measure of change in secondary structure (mean and standard deviation from three individually treated protein samples).

homologous proteins which showed that the main thermal transition in barley LTP1 is in excess of 100°C [24], and the cherry allergen Pru av 3 between 70 and 100°C [25]. Also peach LTP, Pru p 3 showed irreversible denaturation following heating to 95°C [26]. However, the results are in contrast to the findings of Sancho *et al.* who observed only a minor loss of Mal d 3 secondary structure when the protein was heated to 100°C in the absence of oxygen [27]. It seems likely that heating in the presence of oxygen in this study may result in disulphide shuffling, as has been observed for α-lactalbumin [28].

Figure 5A shows the effect of temperature on the amount of immunoreactive Mal d 3 detected by a rabbit polyclonal anti-sera raised to the native protein. There is a clear decrease in antibody recognition of immunoreactive Mal d 3 treated at elevated temperatures which correlates well with the observed decrease in secondary structure (correlation coefficient = 0.944). The effect of pressure at 20°C was minimal (data not shown) but the effect of pressure treatment at 80°C was significant (Fig. 5B). Here a decrease in the amount of immunoreactive Mal d 3 was observed at higher pressures, although the decrease observed was less than that observed at 110°C. There is a small increase in detected immunoreactive Mal d 3 from 500 to 700 MPa which is not consistent with the observed structural changes. The ELISA assays showed a clear correlation between antibody recognition and secondary structure. The small

differences seen as a result of HP processing are consistent with the previous work showing, for example, that thermal denaturation is much more efficient at reducing allergenicity of celery allergens [29]. Thermal denaturation of LTPs has previously been studied in maize and peach [30, 31] and has shown LTP architecture to be quite resilient to thermal processing.

PEF treatments from 0 to 35 kV did not appear to affect the secondary structure of Mal d 3, as shown in Fig. 6. These results are in agreement with the previously published data on other proteins [23].

3.3 Mal d 1b

The secondary structure of the heterologously expressed apple Mal d 1b was similar to that observed for both recombinant and native Bet v 1 [32], a protein with which Mal d 1b shows both high amino acid sequence identity (64%) and conserved

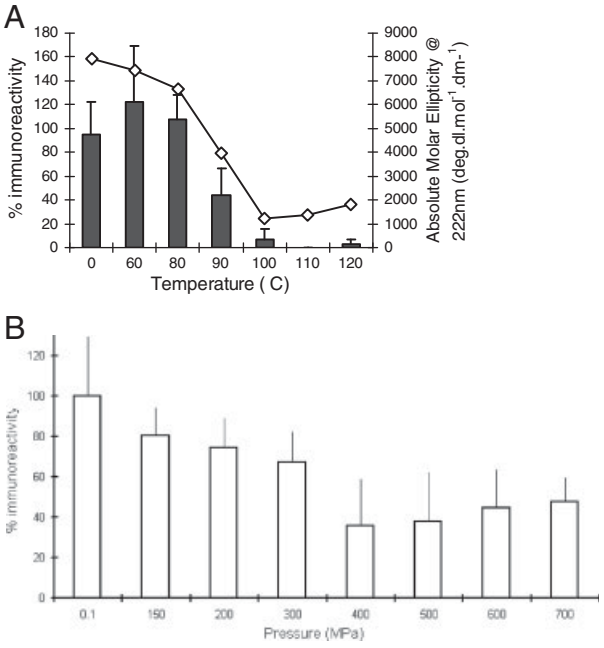

Figure 5. (A) Effect of temperature at ambient pressure on antibody recognition and on α-helix content of Mal d 3. (B) Effect of pressure treatment at 80°C on antibody recognition of Mal d 3.

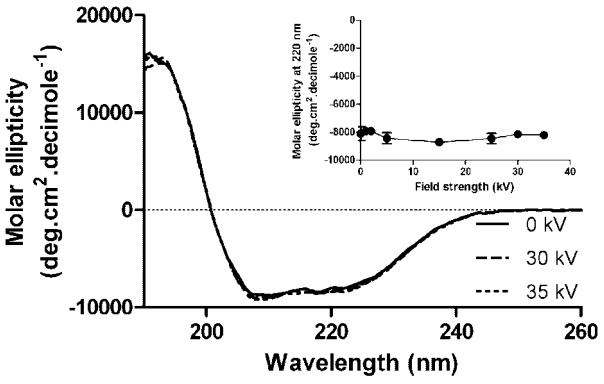

Figure 6. Effect of PEF on the secondary structure of Mal d 3 as determined by CD spectroscopy. Only three spectra are presented for clarity. Graph inset represents molar ellipticity at 220 nm as a measure of secondary structure (mean and standard deviation from three individually treated protein samples).

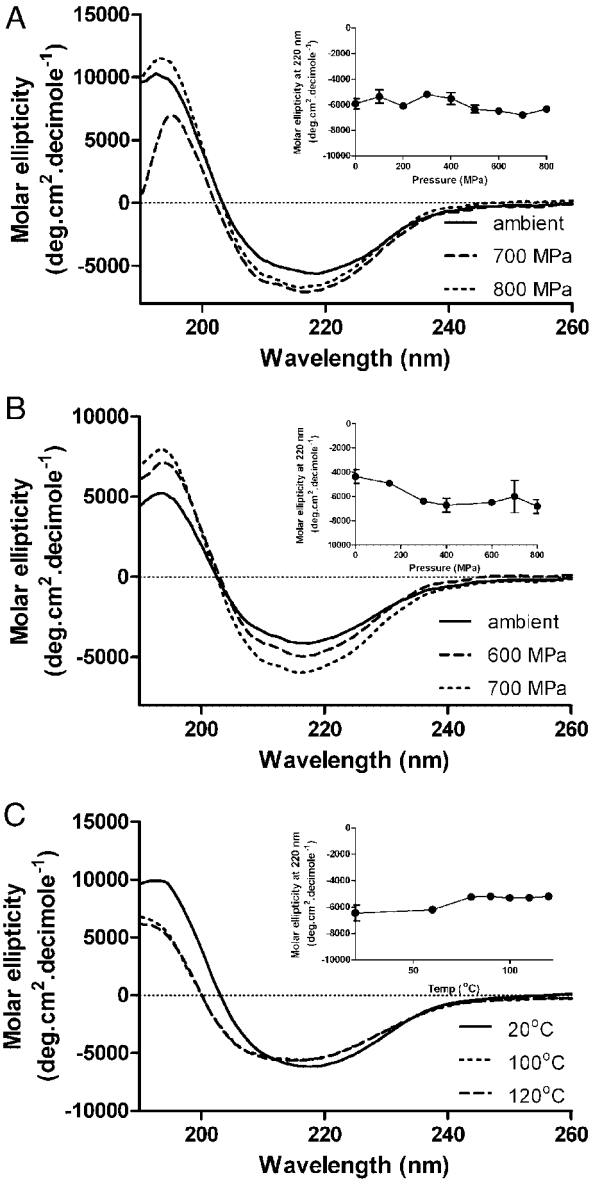

Figure 7. CD Spectra of Mal d 1b after treatment at various pressures at 20°C (A), 80°C (B) and after heating to 20–120°C for 10 min and cooling (C). Only three spectra are presented for clarity. Graph insets represent molar ellipticity at 220 nm as a measure of change in secondary structure (mean and standard deviation from three individually treated protein samples).

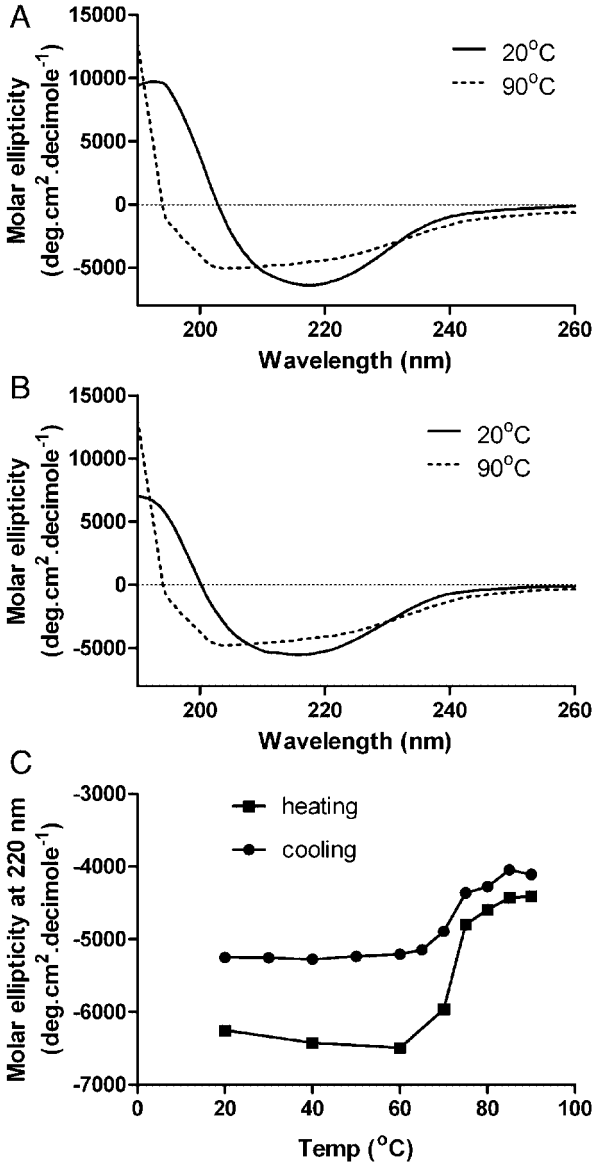

Figure 8. Changes in secondary structure of Mal d 1b with heating and cooling as determined by CD spectroscopy. Heating was performed *in-situ* allowing analysis of transient structural changes. (A) Heating of Mal d 1b (rate of heating was 1°C/min), (B) cooling of Mal d 1b from 90°C (cooling rate was 1°C/min) and (C) change in molar ellipticity at 220 nm during heating (squares) and cooling (circles).

features. Due to the difficulty of purifying Mal d 1b from apple tissue, no published native CD solution spectra exist, however, a similar spectrum has been reported for recombinant Mal d 1a [19]. The observed CD data correlate well with that of Bet v 1 and with the helix/sheet distribution expected from the analysis of the Mal d 1b sequence by PredictProtein (http://www.predictprotein.org) and so it is likely that our heterologously expressed Mal d 1b is folded in a fashion that resembles the native protein.

Mal d 1b showed small changes in secondary structure following high-pressure treatment at 20°C (Fig. 7A) with rather more alteration after high-temperature treatment (Fig. 7B). Pressure treatment of Mal d 1b at 20°C caused a slight, but noticeable, increase in ellipticity as the pressure was increased which became more marked at 80°C (Fig. 7B). However, it seems unlikely that the increase in intensity is a result of an increase in secondary structure. It is possible that the observed increase in ellipticity is due to the formation of intermediate secondary structures, but the available data do not confirm this. Heating of recombinant Mal d 1b to temperatures up to 120°C caused an apparent change in structure as determined by CD (Fig. 7C), but did not irreversibly denature Mal d 1b to a significant extent. The observed change in structure upon heating and cooling is consistent with a loss of β-sheet. CD measurements made while heating *in situ* (Figs. 8A–C) show that significantly more unfolding occurred above 60°C but that the protein partially refolded on cooling.

Meyer-Pittroff *et al.* [33] reported that changes in the secondary structure of recombinant apple Mal d 1 occurred after high-pressure treatment (100–600 MPa), and attributed loss of allergenic potential of apple slices after high-pressure treatment to this structural change. However, the observed loss of Mal d 1 structure was minor and is unlikely to account for the total loss of allergenicity. Unless very subtle changes in allergen structure can cause total loss of IgE-binding capacity it seems likely that matrix-associated effects must play a role in the lack of reactivity of IgE to HP-treated apple tissue. Although it has been shown that a mutation of five residues can lower the allergenicity of Mal d 1 by tenfold [34], the effect on secondary structure of this mutation was not measured, so it is impossible to assess whether the mutation lowered allergenicity by changing the protein conformation.

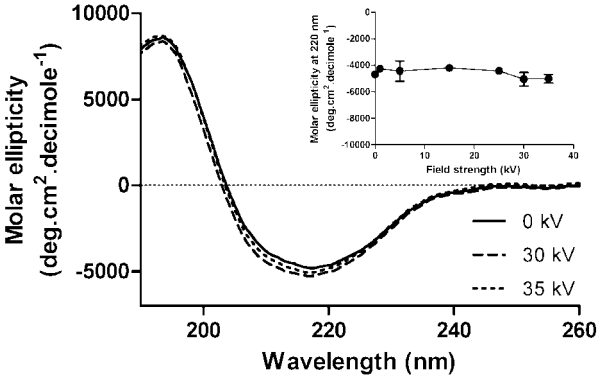

Figure 9. Effect of PEF on the secondary structure of Mal d 1b as determined by CD spectroscopy. Only three spectra are presented for clarity. Graph inset represents molar ellipticity at 220 nm as a measure of secondary structure (mean and standard deviation from three individually treated protein samples).

PEF treatment of Mal d 1b (Fig. 9) resulted in minor increases in ellipticity at higher field strengths (30 and 35 kV), similar to those observed during high-pressure treatment. Again, it is possible that these changes represent formation of intermediate secondary structures. The effects of PEF on protein structure are largely uncharacterized and are heavily dependent on the experimental setup so it is currently impossible to predict treatment effects on proteins. Structural and immunological analyses of more proteins after PEF treatment would be desirable in order to generate sufficient information as to whether allergen structure is likely to change during such treatments, and to demonstrate substantial equivalence for PEF-treated allergen containing foods.

4 Concluding remarks

PEF treatment did not significantly affect the secondary structure of any of the plant allergens in this study. However, the scarcity of studies on the effects of PEF on protein structure in general means that it is difficult to predict whether other allergens may be affected under similar processing conditions. It is, however, difficult to imagine a mechanism by which PEF treatment could directly influence the structure of allergenic proteins. It is more probable that highly localized thermal fluctuations in the treatment cell are responsible for the minor observed structural changes.

High-pressure treatment had little effect on the α-helical structure of the ''prolamin-fold'' proteins Ara h 2 and 6 from peanut or apple Mal d 3 at either room temperature or at 80°C when HPP-induced temperature changes were taken into account. The secondary structure of Mal d 1b, while less stable, was able to refold after treatment and showed no consistent changes after treatment. The changes that were observed could be attributed to changes in aggregation state, which is likely, like other protein–protein interactions, to be concentration dependent. Such studies on purified allergens provide important insights into how different protein scaffolds respond to novel-processing procedures and conditions. The data presented in this report will contribute to the integrative allergenic risk assessment process required before widespread application of such new procedures. It will also help inform future studies to obtain data on how the context of the food matrix may modulate the response of allergens to novel-processing procedures. This is especially important, since other food constituents (proteins, lipids and sugars) and the structure of the food itself may affect the stability properties of allergens, their release and bioaccessiblity in the gastrointestinal tract and their resulting biological activity in the wider human body. Data on such effects are necessary to demonstrate either the equivalence of novel-processing technologies to conventional counterparts or show foods subject to such processes are safe to eat, assessments which they must pass before gaining regulatory approval.

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5 References

[1] Kinefuchi, M., Watanabe, K., Komiya, S., Yamazaki, A. *et al.*, Characteristic of retrogradation of pressure-treated cooked rice. *J. Appl. Glycosci.* 1999, *46*, 31–38.

[2] Sasagawa, A., Yamazaki, A., in: Hayashi, R. (Ed.) *Trends in High-Pressure Bioscience and Biotechnology* Elsevier, Amsterdam 2002, pp. 375–384.

[3] Garcia, D., Gomez, N., Manas, P., Raso, J. *et al.*, Pulsed electric fields cause bacterial envelopes permeabilization depending on the treatment intensity, the treatment medium pH and the microorganism investigated. *Int. J. Food Microbiol.* 2007, *113*, 219–227.

[4] Schilling, S., Alber, T., Toepfl, S., Neidhart, S. *et al.*, Effects of pulsed electric field treatment of apple mash on juice yield and quality attributes of apple juices. *Innovative Food Sci. Emerg. Technol.* 2007, *8*, 127–134.

[5] Guderjan, M., Topfl, S., Angersbach, A., Knorr, D., Impact of pulsed electric field treatment on the recovery and quality of plant oils. *J. Food Eng.* 2005, *67*, 281–287.

[6] Mills, E. N., Jenkins, J. A., Alcocer, M. J., Shewry, P. R., Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. *Crit. Rev. Food Sci. Nutr.* 2004, *44*, 379–407.

[7] Hinrichs, J., Rademacher, B., High pressure thermal denaturation kinetics of whey proteins. *J. Dairy Res.* 2004, *71*, 480–488.

[8] Galazka, V. B., Ledward, D. A., Dickinson, E., Langley, K. R., High pressure effects on emulsifying behavior of whey protein concentrate. *J. Food Sci.* 1995, *60*, 1341–1343.

[9] St John, R. J., Carpenter, J. F., Randolph, T. W., High pressure fosters protein refolding from aggregates at high concentrations. *Proc. Natl. Acad. Sci. USA* 1999, *96*, 13029–13033.

[10] Van Loey, A., Verachtert, B., Hendrickx, M., Effects of high electric field pulses on enzymes. *Trends Food Sci. Technol.* 2001, *12*, 94–102.

[11] Li, Y. Q., Chen, Z. X., Mo, H. Z., Effects of pulsed electric fields on physicochemical properties of soybean protein isolates. *Lwt-Food Sci. Technol.* 2007, *40*, 1167–1175.

[12] Zhang, R. B., Cheng, L., Wang, L. M., Guan, Z. C., Inactivation effects of PEF on horseradish peroxidase (HRP) and pectinesterase (PE). *IEEE Trans. Plasma Sci.* 2006, *34*, 2630–2636.

[13] Radauer, C., Breiteneder, H., Evolutionary biology of plant food allergens. *J. Allergy Clin. Immunol.* 2007, *120*, 518–525.

[14] Lehmann, K., Schweimer, K., Reese, G., Randow, S. *et al.*, Structure and stability of 2S albumin-type peanut allergens:

implications for the severity of peanut allergic reactions. *Biochem. J.* 2006, *395*, 463–472.

[15] Koppelman, S. J., de Jong, G. A. H., Laaper-Ertmann, M., Peeters, K. A. B. M. *et al.*, Purification and immunoglobulin E-binding properties of peanut allergen Ara h 6: evidence for cross-reactivity with Ara h 2. *Clin. Exp. Allergy* 2005, *35*, 490–497.

[16] Jenkins, J. A., Griffiths-Jones, S., Shewry, P. R., Breiteneder, H. *et al.*, Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an in silico analysis. *J. Allergy Clin. Immunol.* 2005, *115*, 163–170.

[17] Fernandez-Rivas, M., Cross-reacting allergens at the molecular scaled a north-south comparison. *Revue Francaise D Allergologie et D Immunologie Clinique* 2006, *46*, 167–169.

[18] Beuning, L. L., Bowen, J. H., Persson, H. A., Barraclough, D. *et al.*, Characterisation of Mal d 1-related genes in Malus. *Plant Mol. Biol.* 2004, *55*, 369–388.

[19] Oberhuber, C., Ma, Y., Marsh, J., Rigby, N. *et al.*, Purification and characterisation of relevant natural and recombinant apple allergens. *Mol. Nutr. Food Res.* 2008, *52*, S208–S219.

[20] Marsh, J., Rigby, N., Wellner, K., Reese, G. *et al.*, Purification and characterisation of a panel of peanut allergens suitable for use in allergy diagnosis. *Mol. Nutr. Food Res.* 2008, *52*, S272–S285.

[21] De Roeck, A., Duvetter, T., Fraeye, I., Van der Plancken, L. *et al.*, Effect of high-pressure/high-temperature processing on chemical pectin conversions in relation to fruit and vegetable texture. *Food Chem.* 2009, *115*, 207–213.

[22] Toepfl, S., Pulsed electric fields (PEF) for permeabilization of cell membranes in food- and bioprocessing – applications, process and equipment design and cost analysis, PhD Thesis, Berlin University of Technology, 2006.

[23] Barsotti, L., Dumay, E., Mu, T. H., Diaz, M. D. F. *et al.*, Effects of high voltage electric pulses on protein-based food constituents and structures. *Trends Food Sci. Technol.* 2001, *12*, 136–144.

[24] Lindorff-Larsen, K., Winther, J. R., Surprisingly high stability of barley lipid transfer protein, LTP1, towards denaturant, heat and proteases. *FEBS Lett.* 2001, *488*, 145–148.

[25] Scheurer, S., Lauer, I., Foetisch, K., San Miguel, M. M. *et al.*, Strong allergenicity of Pru av 3, the lipid transfer protein from cherry, is related to high stability against thermal processing and digestion. *J. Allergy Clin. Immunol.* 2004, *114*, 900–907.

[26] Gaier, S., Marsh, J., Oberhuber, C., Rigby, N. M. *et al.*, Purification and structural stability of the peach allergens Pru p 1 and Pru p 3. *Mol. Nutr. Food Res.* 2008, *52*, S220–S229.

[27] Sancho, A. I., Rigby, N. M., Zuidmeer, L., Asero, R. *et al.*, The effect of thermal processing on the IgE reactivity of the non-specific lipid transfer protein from apple, Mal d 3. *Allergy* 2005, *60*, 1262–1268.

[28] Wijesinha-Bettoni, R., Gao, C., Jenkins, J. A., Mackie, A. R. *et al.*, Heat treatment of bovine alpha-lactalbumin results in partially folded, disulfide bond shuffled states with enhanced surface activity. *Biochemistry* 2007, *46*, 9774–9784.

[29] Jankiewicz, A., Baltes, W., Bogl, K. W., Dehne, L. I. *et al.*, Influence of food processing on the immunochemical stability of celery allergens. *J. Sci. Food Agric.* 1997, *75*, 359–370.

[30] Scibilia, J., Pastorello, E. A., Zisa, G., Ottolenghi, A. *et al.*, Maize food allergy: a double-blind placebo-controlled study. *Clin. Exp. Allergy* 2008, *38*, 1943–1949.

[31] Brenna, O., Pompei, C., Ortolani, C., Pravettoni, V. *et al.*, Technological processes to decrease the allergenicity of peach juice and nectar. *J. Agric. Food Chem.* 2000, *48*, 493–497.

[32] Faber, C., Lindemann, A., Sticht, H., Ejchart, A. *et al.*, Secondary structure and tertiary fold of the birch pollen allergen Bet v 1 in solution. *J. Biol. Chem.* 1996, *271*, 19243–19250.

[33] Meyer-Pittroff, R., Behrendt, H., Ring, J., Specific immunomodulation and therapy by means of high pressure treated allergens. *High Press. Res.* 2007, *27*, 63–67.

[34] Bolhaar, S. T., Zuidmeer, L., Ma, Y., Ferreira, F. *et al.*, A mutant of the major apple allergen, Mal d 1, demonstrating hypo-allergenicity in the target organ by double-blind placebo-controlled food challenge. *Clin. Exp. Allergy* 2005, *35*, 1638–1644.

