

Original article

The effect of thermal processing on the IgE reactivity of the non-specific lipid transfer protein from apple, Mal d 3

Background: Non-specific lipid transfer proteins (LTPs) are involved in allergy to fresh and processed fruits. We have investigated the effect of thermal treatment and glycation on the physico-chemical and IgE-binding properties of the LTP from apple (Mal d 3).

Methods: Mal d 3 was purified from apple peel and the effect of heating in the absence and presence of glucose investigated by CD spectroscopy, electrospray and MALDI-TOF mass spectrometry. IgE reactivity was determined by RAST and immunoblot inhibition, SPT and basophil histamine release test.

Results: The identity and IgE reactivity of purified Mal d 3 was confirmed. Mild heat treatment (90°C, 20 min) in the absence or presence of glucose did not alter its IgE reactivity. More severe heat treatment (100°C, 2 h) induced minor changes in protein structure, but a significant decrease in IgE-binding (30-fold) and biological activity (100- to 1000-fold). Addition of glucose resulted in up to four glucose residues attached to Mal d 3 and only a 2- and 10-fold decrease of IgE-binding and biological activity, respectively.

Conclusions: Only severe heat treatment caused a significant decrease in the allergenicity of Mal d 3 but glycation had a protective effect. The presence of sugars in fruits may contribute to the thermostability of the allergenic activity of LTP in heat-processed foods.

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Lipid transfer proteins (LTP) have been identified as allergens in various *Rosaceae* fruits including apple, peach, apricot, plum and cherry (1) and found to be highly homologous (2). In addition, immunological cross-reactivity between LTPs from many botanically unrelated fruits and vegetables has been reported (3, 4). As members of the prolamin superfamily (5), LTPs share a conserved cysteine skeleton with four intra-molecular disulphide bridges stabilizing a bundle of four α -helices and a C-terminal coil (6, 7). This compact structure makes LTP highly resistant to proteolytic attack and to food processing. IgE reactivity of proteins can be unchanged, decreased or increased during food processing (8). LTPs retain their allergenic properties in

thermally-treated products such as purees, nectars and juices (9) and polenta (10), indicating the heat stability of purified LTPs extends to the whole food (11). In addition to protein unfolding and aggregation, various chemical modifications can occur during processing, one of the most important being the Maillard reaction (12). Maillard adducts can modify the IgE reactivity of peanut (13) and cherry allergens (14, 15) however the basis of the effects of Maillard reactions on allergenicity of proteins are poorly characterized at a molecular level. This is essential if food manufacturers are to move towards knowledge-based ways of managing allergen risks during processing and to inform the allergenic risk assessment process, which forms part of the regulatory framework pertaining to novel foods and processes. In addition, well-defined and well-characterized allergens are needed for a precise, rapid and reliable detection of hypersensitivity to this allergen in complex food matrix (16) and for future immunotherapy (17, 18). This report describes a molecular study of the effect of heating, (including glycation), on the physico-chemical and IgE-binding properties of the LTP from apple, Mal d 3.

Abbreviations: Da, Dalton; DBPCFC, double blind placebo control food challenge; EDTA, ethylenediaminetetraacetic acid; g₁Mal d 3, thermally treated Mal d 3 at 90°C for 20 min in presence of sugar; g₂Mal d 3, thermally treated Mal d 3 at 100°C for 2 h in presence of sugar; h₁Mal d 3, thermally treated Mal d 3 at 90°C for 20 min; h₂Mal d 3, thermally treated Mal d 3 at 100°C for 2 h; LTP, non-specific lipid transfer protein; OAS, oral allergy syndrome; RAST, radio allergosorbent test; SPT, skin prick test.

Materials and methods

Patients

Twenty-two patients from Italy (Clinica San Carlo, Paderno Dugnano) and Spain (Fundación Hospital Alcorcón, Alcorcón) were selected on the basis of a clinical history of allergy to apple (mainly OAS) and a positive prick-to-prick test with fresh apple. None of these patients suffered from birch pollen allergy and all had a negative SPT with commercial birch pollen extract (Allergopharma, Reinbeck, Germany). In addition, all patients demonstrated IgE reactivity to LTP as judged by RAST with purified Mal d 3 or by SPT with a commercial peach extract containing Pru p 3 but no demonstrable Pru p 1 (ALK-Abello, Lainate, Italy). Such commercial fruit extracts have been shown to be devoid of birch pollen-related allergen activity (Bet v 1 and 2 homologues) and rich in LTP and can consequently be used to detect LTP-reactivity (19). As negative controls for LTP sensitization, a group of 11 patients from Italy (Paderno Dugnano) with a clinical history of birch pollen-related apple allergy, a positive SPT with birch pollen extract and a positive prick-to-prick test with fresh apple was selected. For some experiments where larger serum quantities were needed, two individual reference sera (pf194 and pf227) and a reference serum pool with established IgE reactivity to Mal d 3 were used. These serum samples contained 7.9, 5.2 and 19.2 IU/ml of IgE against Mal d 3, respectively, as determined by RAST.

Skin prick test

The Skin prick tests (SPTs) were performed according to Dreborg and Frew (20) with purified natural Mal d 3. Lyophilized Mal d 3 was reconstituted at 1 mg/ml in 0.1 M NaCl, 0.03 M NaHCO₃, followed by dilution in the same buffer supplemented with 50% (v/v) glycerol and 0.4% (w/v) phenol to final concentrations of 50.0, 10.0, 2.0 and 0.4 µg/ml of Mal d 3. In addition, SPTs were performed with Mal d 3 (50 µg/ml) heated at 100°C for 60 min. A wheal diameter of <3 mm was regarded as negative. The SPT procedure did not need specific authorization by an ethical committee as it was carried out using natural extracts. Every patient gave an informed written consent before SPT were performed.

Thermal treatment of Mal d 3

The Mal d 3 was purified from apple peel (cv. Golden Delicious) extracts by ammonium sulphate precipitation (95% saturation), cation-exchange chromatography (Poros HS column, Perspective Biosystems, Frammingham, USA) and size-exclusion chromatography (Superdex 75, Amersham Biosciences, Buckinghamshire, UK). Purity was assessed by SDS-PAGE analysis. Purified Mal d 3 (0.70 mg/ml) in HPLC grade water (Barnstead NANOpure Diamond, Triple Red, Buckinghamshire, UK) was heated at 90°C for 20 min in a thermostatically controlled heating block (QBT1, Grant, Cambridge, UK) in the presence (g₁Mal d 3) or absence (h₁Mal d 3) of β-D-glucose (30% w/w). Unreacted sugars were removed by dialysis against HPLC grade water using 1 kDa cut-off tubing (DisposoDialyzer, Spectrum Labs). A more severe heat treatment was performed in the same manner but using 1.77 mg/ml Mal d 3 flushed with argon prior to heating at 100°C for 2 h in the presence (g₂Mal d 3) or absence (h₂Mal d 3) of β-D-glucose (30% w/w). Native and modified Mal d 3 samples were analyzed by HPLC-electrospray mass spectrometry as described by Moreno et al. (21).

Circular dichroism (CD) spectroscopy

Far-ultraviolet (UV) CD spectra (300–190 nm) of Mal d 3 (0.34 mg/ml in 20 mM sodium phosphate buffer, pH 7.0) were performed using a Jasco J-710 Spectropolarimeter (Jasco, Tokyo, Japan) and converted to molar ellipticity according to Moreno et al. (21). Spectra were recorded during heating (1°C/min) from 20 to 95°C at intervals of 5°C after 3 min thermal equilibrium and during the cooling phase at 0.5°C/min.

SDS-PAGE, immunoblotting and immunoblot inhibition

SDS-PAGE was performed under reducing conditions using a 12% Bis-Tris gel in a NuPAGE system (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. Proteins were visualized by Colloidal Coomassie staining (Invitrogen). For immunoblotting apple extract proteins (10 µg/cm) were separated under reducing conditions using a NuPAGE 4–12% Bis-Tris gel and blotted as described by Bolhaar et al. (22). For blot inhibition studies, patient serum was preincubated with 150 µl of Mal d 3 (5 µg) for 30 min. Protein was determined using the bicinchoninic acid assay (Pierce, Rockford, IL) with bovine serum albumin as a standard (23).

Following SDS-PAGE analysis, native Mal d 3 was subjected to proteomic analysis using in-gel trypsin digestion and tryptic peptides were analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) at the joint Institute of Food Research-John Innes Center (IFR-JIC) proteomics facility as described by Moreno et al. (21).

Radio-allergosorbent test (RAST) and RAST-inhibition

RAST was performed as described previously (24). For RAST inhibition, serum was preincubated with 50 µL of serial dilutions of inhibitor (Mal d 3, h₁Mal d 3, h₂Mal d 3, g₁Mal d 3 and g₂Mal d 3) in PBS containing 0.3% (w/v) BSA and 0.1% (v/v) Tween 20 for 2 h prior to addition of Sepharose-coupled allergen (apple extract or Mal d 3).

Basophil histamine release assay (BHR)

White blood cells were isolated from blood of a non-allergic donor by Percoll centrifugation and stripped from IgE by lactic acid treatment as described elsewhere (25, 26). Subsequently cells were resensitized with patients' serum. Histamine release was performed with purified Mal d 3 and heat-treated versions at concentrations from 1 pg/ml to 100 ng/ml. Released histamine was measured by the fluorometric method essentially as described by Siraganian (27). Stripped cells were used as a negative control and induced a histamine release lower than 3%. The protocol was approved by the medical ethical committee (MEC) of the Amsterdam Medical Center under project number MEC 97/030.

Results

Physico-chemical characterization of Mal d 3

Proteomic analysis of the purified protein identified all peptide masses as apple LTP (Accession number: Q9M5X7) with a probability of identification of 100%

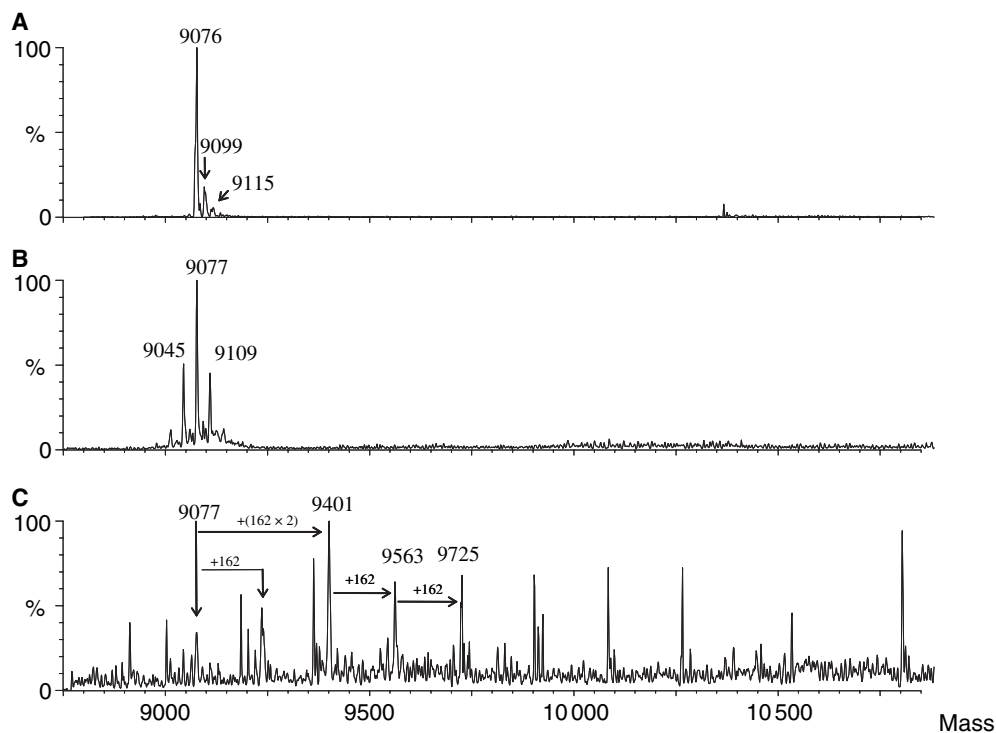


Figure 1. Electrospray mass spectrometry. Mass spectra of purified Mal d 3 (A), h₂Mal d 3 (B) and g₂Mal d 3 (C). Transformed data from original mass spectra containing multiply charged ions using the MaxEnt algorithm to extract the masses of the component molecules. Peaks have been normalized so that the intensity of the larger peak is equal to 100%. The responses do not reflect the relative intensities of ions. The mass spectra have been annotated to show major molecular masses.

based on molecular weight search (MOWSE) score. The ES-MS analysis of purified Mal d 3 gave a major component with of 9076 Da (Fig. 1A), and minor peaks at 9099 and 9115 Da corresponding to the $(M + Na)^+$ and $(M + K)^+$ adducts respectively, in agreement with the mass of Mal d 3 previously reported by Sanchez-Monge et al. (1).

IgE reactivity of purified Mal d 3

Thirteen of the twenty-two patients with established IgE reactivity to LTP were subjected to titrated SPT with purified Mal d 3 (Fig. 2). All were positive at 50 μ g/ml with diameters between 4 and 11 mm whilst 10 and 2 μ g/ml Mal d 3 were negative in 4/13 patients (31%) and 11/13 patients (84%), respectively. All patients had a negative SPT at 0.4 μ g/ml Mal d 3. None of the control patients with birch pollen-related apple allergy ($n = 11$) had a positive SPT to Mal d 3, demonstrating its specificity for diagnosing LTP-related apple allergy.

Sera from seven LTP-sensitized patients were used for apple immunoblot analysis (Fig. 3A). All displayed significant IgE-binding to a band at the expected M_r 9000 of Mal d 3, although two sera displayed only borderline reactivity. The identification of this band as LTP was confirmed by inhibition with purified Mal d 3

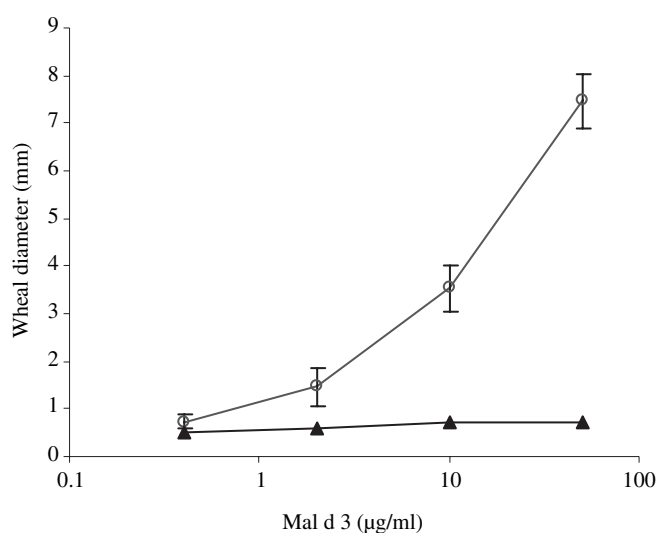


Figure 2. Skin prick test. LTP-related apple allergic patients ($n = 13$) ($\circ$). Birch pollen-related apple allergic patients ($n = 11$) ($\blacktriangle$). Mean values and standard deviation bars are represented.

(5 μ g per lane). Serum samples from lanes 2, 4 and 6 were also used for RAST inhibition. The apple RAST was inhibited up to 95% by purified Mal d 3 (Fig. 3B).

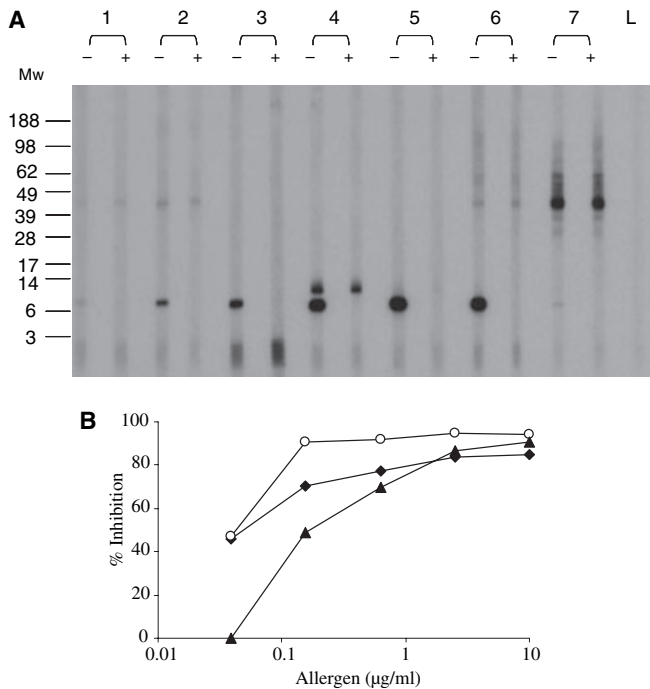


Figure 3. Immunoblot inhibition (A). Apple extract inhibition by purified Mal d 3 using sera of seven apple allergic patients (lanes 1–7). Lanes: (–) no inhibitor; (+) with inhibitor; L, labeled sheep anti-human IgE control. RAST inhibition studies (B). Apple extract inhibition by purified Mal d 3 using sera from patient 2 (○), 4 (■) and 6 (▲).

Physico-chemical characterization of Mal d 3 on heating

Prior to heating, native Mal d 3 gave a CD spectrum characteristic of an α -helical structure with a double negative minimum at 208 and 222 nm and a maximum at 195 nm (Fig. 4A). During heating the intensity of the negative peak decreased, indicative of a small change in Mal d 3 α -helical secondary structure, the protein returning to its native state on cooling (Fig. 4A,B). Whilst mild heating (90°C, 20 min) of Mal d 3 alone (h_1 Mal d 3) did not cause any changes to the mass of the protein, a more extensive heat treatment (h_2 Mal d 3) resulted in the appearance of two additional masses indicative of a mass loss of 32 (9045 Da) or gain (9109 Da) compared with the native protein (Fig. 1B). These additional masses might correspond to oxidation of a disulphide bond by molecular oxygen accounting for the mass gain, and to nucleophilic attack with the elimination of sulphur dioxide and a mass decreased by 32 Da compared to the native LTP.

Mild heating (90°C, 20 min) in the presence of glucose (g_1 Mal d 3) resulted in a small proportion of monoglycated Mal d 3 being formed, the majority of the protein remaining unmodified (data not shown). However, more severe heating (100°C, 2 h) (g_2 Mal d 3), produced both an increase in the complexity of the mass spectrum, and a significant decrease in the signal intensity, most likely due

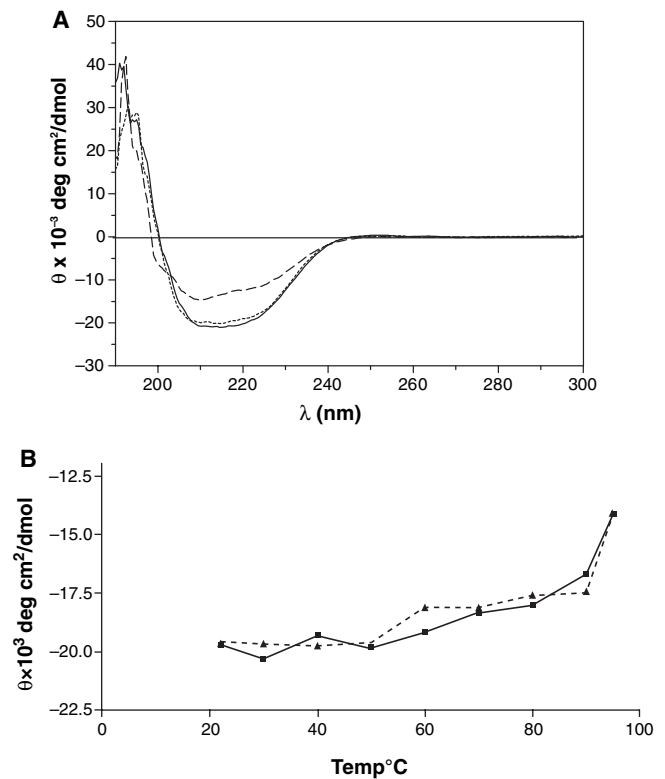


Figure 4. Effect of heating on Mal d 3 secondary structure. (A) Far-UV CD spectra of Mal d 3 at 20°C (—), 95°C (---) and cooled to 20°C (...). (B) Thermal transition curve of Mal d 3 recorded at 208 nm after heating (—) and cooling (---).

to the effect of glycation on the ionization of the protein. Nevertheless, it was possible to identify a peak of 9076 Da corresponding to the native protein and other peaks at higher masses with an increase of 162 Da corresponding to the addition of between 1 and 4 anhydro-glucose residues (9239, 9401, 9563, 9725 Da) (Fig. 1C). None of the thermal treatments resulted in the formation of oligomers of Mal d 3 as determined by size exclusion chromatography (data not shown).

IgE reactivity and basophil histamine release (BHR) of heated Mal d 3

Two Mal d 3-reactive reference sera (pf194 and pf227) and the reference serum pool were used for RAST inhibition experiments to assess the influence of heat treatment on the IgE-binding potency of Mal d 3. Mild heat treatment did not alter the inhibitory potency of Mal d 3, with native protein, h_1 Mal d 3 and g_1 Mal d 3 (data not shown). However, whilst more severely heat-treated h_2 Mal d 3 and g_2 Mal d 3 could still completely inhibit IgE binding to Mal d 3, inhibitory potencies were significantly reduced (Fig. 5). Thus, the concentration for half-maximal inhibition (IC_{50}) increased from 0.07 μ g/ml for native Mal d 3, to 0.15 μ g/ml for g_2 Mal d 3 and to 1–2 μ g/ml for h_2 Mal d 3.

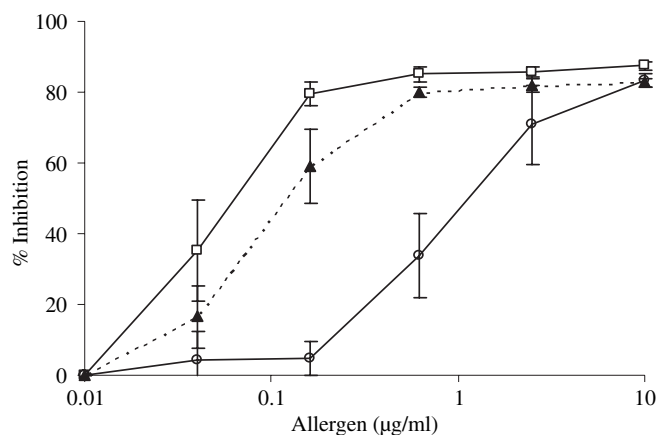


Figure 5. RAST inhibition studies with thermally treated Mal d 3. Mal d 3 coupled to sepharose with inhibitor as follows: Mal d 3 (□), h₂Mal d 3 (○) and g₂Mal d 3 (▲). Mean values and standard deviation bars of two sera and a serum pool are represented.

The biological activity of thermally modified Mal d 3 was analyzed by BHR using two Mal d 3-reactive sera. A dose-dependent release of histamine was obtained for Mal d 3. Even at the lowest dose of Mal d 3 (1 pg/ml), significant mediator release was observed (up to 27%), illustrating the allergenicity of LTP (Fig. 6A,B). In line with the RAST-inhibition results, both h₁Mal d 3 and g₁Mal d 3 demonstrated similar biological activity to the native allergen (Fig. 6A). These results are consistent with the fact that all patients showed the same SPT reactivity both with native Mal d 3 and h₁Mal d 3. Severe heat treatment did modify the capacity of the treated protein to induce histamine release showing between 100- and 1000-fold decrease for h₂Mal d 3. Addition of glucose during heat treatment (g₂Mal d 3) also had an effect on the biological activity of Mal d 3, with only a 10-fold reduction in its potency to induce histamine release compared to that of native Mal d 3 (Fig. 6B).

Discussion

Mild heat treatment (90°C, 20 min) of native Mal d 3 did not significantly alter its IgE binding capacity or its ability to trigger mediator release. This is probably because the unfolding of Mal d 3 is reversible after mild heating, as has been described for the LTP from barley (28). The presence of four intra-molecular disulphide bonds most likely assists in the refolding process. More severe thermal treatment (100°C, 2 h) resulted in the appearance of two additional masses, which could not unequivocally be assigned. Although efforts were made to exclude oxygen during heating, traces are likely to be still present. The additional masses might therefore be explained by oxidation of a disulphide bond by molecular oxygen. Such partial oxidation would account for the appearance of an

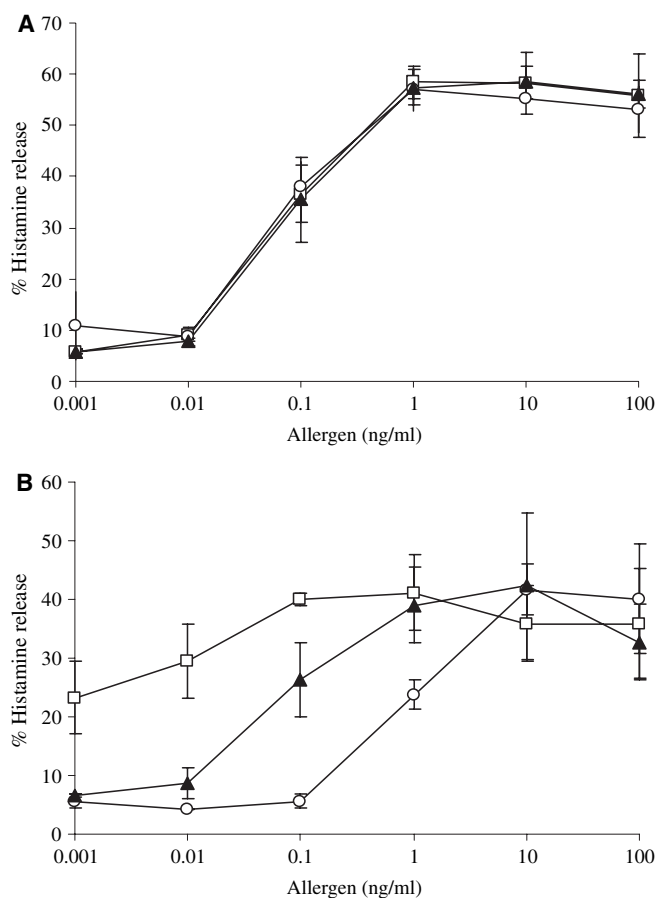


Figure 6. Effect of thermal modification of Mal d 3 on histamine release. Mild heat treatment (90°C, 20 min) (A) and severe heat treatment (100°C, 2 h) (B). Mal d 3 (□), h₂Mal d 3 (○) and g₂Mal d 3 (▲). Mean values and standard deviation bars of two sera are represented.

LTP molecule with a mass increased by 32 Da. In turn, this molecule has the potential, under nucleophilic attack, to eliminate sulphur dioxide giving rise to the other molecular mass observed, with a thioether bond replacing the disulphide bond, and a mass decreased by 32 Da compared to the native LTP. Such modifications would most likely affect refolding of Mal d 3 upon cooling, explaining the reduced IgE binding potency of h₂Mal d 3. Molecular dynamics simulations of LTPs have indicated that indeed one disulphide bond is less stable and consequently probably more susceptible to oxidation (29). Loss of a disulphide bond would mean that native folding would not be fully recovered during cooling, affecting conformational but not linear epitopes and hence significantly reducing (30-fold), but not completely abolishing, the IgE binding capacity of the protein. Alternatively, a fraction of the LTP molecules remain unmodified and hence can fully refold, which would account for the reduced but complete inhibitory potency. The observation that the inhibition curves of native Mal d 3 and h₂Mal d 3 have very similar shapes might favor the latter explanation.

Apple-based products represent ideal conditions for Maillard reactions due to the presence of sugars and the high temperatures used during processing. In this reaction the anomeric carbon reacts with the free β -amino groups of lysine residues forming a Schiff's base and later the corresponding ketoamine (12). We observed minimal glycation following mild heating, indicating that mild thermal treatments, such as UHT where juices are rapidly heated to 135–140°C for 2–5 s, or the dearomatization procedures (110°C for 1–70 s) used during fruit juice manufacture, might not modify Mal d 3 structure. Severe heat treatment also only induced limited glycation, without aggregation or rearrangement of the sugars to cross-link the Mal d 3. Intriguingly, glycation appeared to protect the IgE binding capacity of Mal d 3 compared to h₂Mal d 3. Based on the 3D structure of maize LTP (6) and the peach LTP model (7), all four lysines of Mal d 3 (Q9M5X7) are likely to be surface accessible, representing potential sites of non-enzymatic glycation. The electrospray results are consistent with this, with up to four glucose adducts being found on the Mal d 3. A molecular model of the homologous peach LTP shows that the lysine residues are located in α -helical regions of the protein, which from our CD studies may unfold to a limited extent at high temperature, and suggest that glycation may stabilize the protein conformation at these sites. Since homologous regions of Mal d 3 corresponding to two of the major peach LTP IgE-epitopes identified by Garcia-Casado et al. (7) do not contain lysine residues, glycation would not be expected to dramatically alter IgE reactivity of the apple/peach allergic patients from this study.

The observations that thermally processed foods such as cooked apples (11), commercial peach nectars (9), polenta (10) and cooked cherries (15) are still able to elicit allergic reactions in individuals sensitized towards LTPs is consistent with our observation that glycation has only a moderate effect (2-fold) on IgE-binding although the biological activity showed a 10-fold reduction. Unfortunately, SPT could not be performed with severely heated Mal d 3 due to a lack of material. However, these results maybe clinically relevant since a significant number of patients from this study tolerated processed apple products. Mal d 3 behaved in a contrasting fashion to the recombinant LTP allergen of cherry, Pru av 3, to which IgE reactivity was not diminished (15). However, glycation of the Bet v 1 allergen from cherry, Pru av 1, also reduced IgE binding by 1- to 2-fold although the biological relevance of this was not determined (14). The way in which processing modifies allergenicity is clearly complex. This hampers the development of simple strategies to reduce allergenicity of food products by heat treatment. Greater understanding of its impact at a molecular level will be essential for industry to develop knowledge-based means of reducing allergenicity in the future.

Acknowledgments

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